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PRINCIPAL INVESTIGATOR: Timothy J. Yen, Ph.D.

CONTRACTING ORGANIZATION: Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

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Timothy J. Yen, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111

E-Mail: timothy.yen@fccc.edu

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Drugs that inhibit microtubule functions are one of many anti-neoplastic drugs that are used to combat breast and other cancers. Taxol and vincristine are microtubule poisons that block the proper function of microtubules that are essential for a broad spectrum of motile biological processes that include cell division, vesicle transport, cell shape, and flagella functions. For rapidly proliferating cancer cells, anti-microtubule drugs offers a highly effective means to block cell division and thus stop tumor growth. Nevertheless, these drugs block other microtubule dependent processes that adversely affect the functions of many non-dividing cells. Furthermore, there is the complication that the cancer cells can develop multi-drug resistance that makes them refractile to conventional anti-neoplastic agents. The identification of novel drugs with increased selectivity towards mitotic processes and act synergistically with existing anti-microtubule drugs should enhance and refine the modalities used to treat breast cancer patients. Our interest in the molecular and biochemical mechanisms that are central to mitosis in human cells has led to the identification of novel proteins and pathways that are suited for designing highly specific anti-mitotic drugs. The objective of this proposal is to disrupt such pathways in established breast cancer cell lines to validate them as suitable targets for developing new anti-mitotic drugs.

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Validate Mitotic Checkpoint and Kinetochore Motor Proteins in Breast Cancer Cells as Targets for the Development of Novel Anti-Mitotic Drugs.

Introduction:

Drugs that inhibit microtubule functions are one of many anti-neoplastic drugs that are used to combat breast and other cancers. Taxol and vincristine are microtubule poisons that block the proper function of microtubules that are essential for a broad spectrum of motile biological processes that include cell division, vesicle transport, cell shape, and flagella functions. For rapidly proliferating cancer cells, anti-microtubule drugs offers a highly effective means to block cell division and thus stops tumor growth. Nevertheless, these drugs block other microtubule dependent processes that adversely affect the functions of many non-dividing cells. Furthermore, there is the complication that the cancer cells can develop multi-drug resistance that makes them refractile to conventional anti-neoplastic agents. The identification of novel drugs with increased selectivity towards mitotic processes and act synergistically with existing anti-microtubule drugs should enhance and refine the modalities used to treat breast cancer patients. Our interest in the molecular and biochemical mechanisms that are central to mitosis in human cells has led to the identification of novel proteins and pathways that are suited for designing highly specific anti-mitotic drugs. The objective of this proposal is to disrupt such pathways in established breast cancer cell lines to validate them as suitable targets for developing new anti-mitotic drugs.

Body:

We proposed to manipulate two pathways that are known to be essential and operate only in mitosis of human cells to validate them as suitable targets for the development of novel anti-neoplastic agents. One pathway is specified by the kinesin-like motor protein CENP-E that is essential for aligning chromosomes at the spindle equator during mitosis. The second pathway is a checkpoint pathway that is specified by multiple proteins to ensure cells do not prematurely exit mitosis in the presence of unaligned chromosomes. We proposed four tasks to achieve our goals. We have chosen to analyze three established breast cancer lines and compare their responses to the Hela cervical carcinoma cell line, with which we have studied these two pathways extensively.

Task 1. Evaluate expression of mitotic proteins CENP-E and checkpoint proteins in established breast cancer lines.

We have conducted immunoblot analysis to determine the expression of CENP-E and the checkpoint proteins, hBUB1, hBUBR1, MAD1, MAD2 and Cdc20 in MCF7, T47D and MDA468 cells. All of these proteins were found to be expressed in these cell lines and thus confirmed that they are valid in vivo targets (data not shown). We have determined that all of these proteins are localized to kinetochores in MCF7 and MDA-468 cells. Figures 1 and 3 show localization of hBUB1 and CENP-E to kinetochores of mitotic MCF7 and MDA468 cells, respectively. The presence of CENP-E and various checkpoint proteins at kinetochores support our prediction that these proteins provide similar functions in mitosis as we have shown in Hela cells.

We have also examined the response of MCF7 and MDA468 cells to the microtubule inhibitor, nocodazole and found that this drug will delay cells in mitosis. These findings indicate that the mitotic checkpoint pathway is likely to be intact in these cancer cell lines. Thus, the various checkpoint proteins that we proposed to analyze in this project are strong candidates with which we can use to inhibit this pathway.

Our studies of T47D are lagging because these cells grow at only half the rate as the other cell lines. This unavoidable technical problem has prevented us from conducting all of our

studies simultaneously. We therefore intend to continue to study this cell line independently of MCF7 and MDA468.

Task 2. Evaluate response of T47D, MCF-7 and MDA-MB-468 cells to inhibition of the mitotic checkpoint proteins, hBUBR1, hBUB3, cdc20 and MAD2.

As we have confirmed that these breast cancer lines express the target mitotic checkpoint proteins, we have initiated efforts to inhibit the mitotic checkpoint. We had originally proposed to accomplish this by microinjecting antibodies and overexpression of dominant negative mutants. However, new advances in silencing gene expression by RNA interference (RNAi) have altered our original strategy.

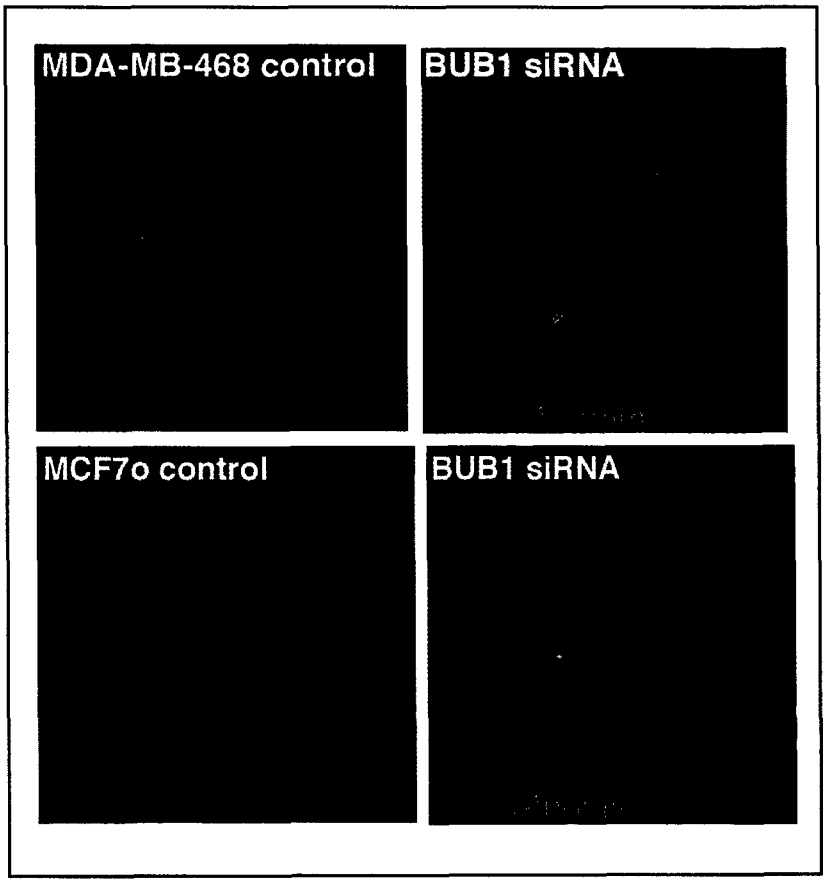

Figure 1. MDA468 and MCF cells were trans-fected with a control (left panels) or BUB1 (right panels) siRNAs and mitotic cells were stained with rabbit anti-hBub1, mouse anti-tubulin and DNA. Images were captured with a 63X oil objective and then pseudo-colored and merged.

Using siRNA, we have successfully inhibited the expression of hBUB1 kinase in Hela cells. One unexpected finding was that the loss of hBUB1 prevented the assembly of MAD1, MAD2 and hBUBR1 checkpoint proteins to the kinetochore (data not shown). Thus, inhibition of hBUB1 kinase may result in the inhibition of multiple checkpoint proteins. Based on these studies, we have transfected MCF7 and MDA468 cells with hBUB1 siRNA. At the single cell level, it is clear that hBUB1 expression can be reduced by siRNA (Figure 1). However, the low transfection efficiencies of these cell lines have made it difficult to interpret results from clonogenic experiments. While there are instances where cells transfected with hBUB1 siRNA exhibited reduced efficiency of colony formation (Figure 2), this outcome is highly variable.

We attribute this to the variability in transfection efficiencies of MCF7 and MDA468 cells. To overcome this obstacle, we plan to infect cells with a recombinant lentivirus that express the siRNA of interest. This viral delivery system was developed to overcome problems with poor transfection efficiencies. We are in the process of making the appropriate constructs so that we may generate large stocks of recombinant lentivirus for the clonogenic studies.

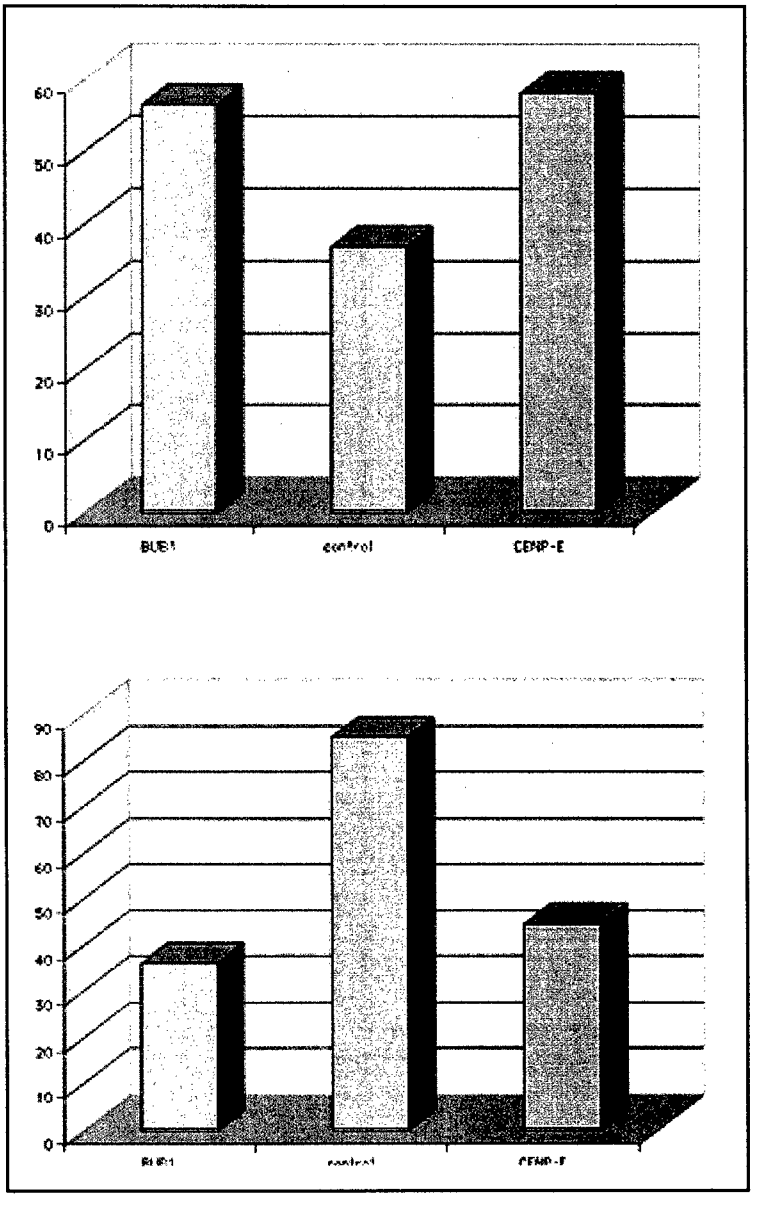

Figure 2. MCF7 (top panel) and MDA-468 (bottom panel) cells were transfected with BUB1 (left), control (center) and CENP-E (right) siRNAs and were plated at approximately 200 cells per 35cm plate. Colonies were stained and counted on day 15 for MCF7 and day 11 for MDA468. Left axis represents colony number.

Task 3. Evaluate CENP-E as a target to block T47D, MCF-7 and MDA-MB-468 cells in mitosis.

As with our studies of the checkpoint pathway, we have opted to inhibit CENP-E function by RNAi technology. Using Hela cells as a positive control, we succeeded to inhibit expression of CENP-E and cells arrest in mitosis because chromosomes fail to align properly (data not shown). As before, we are able to reduce CENP-E expression in MCF7 and MDA468 cells at the single cell level. We are therefore also generating recombinant lentivirus that express CENP-E RNAi so that we may conduct our clonogenic survival studies.

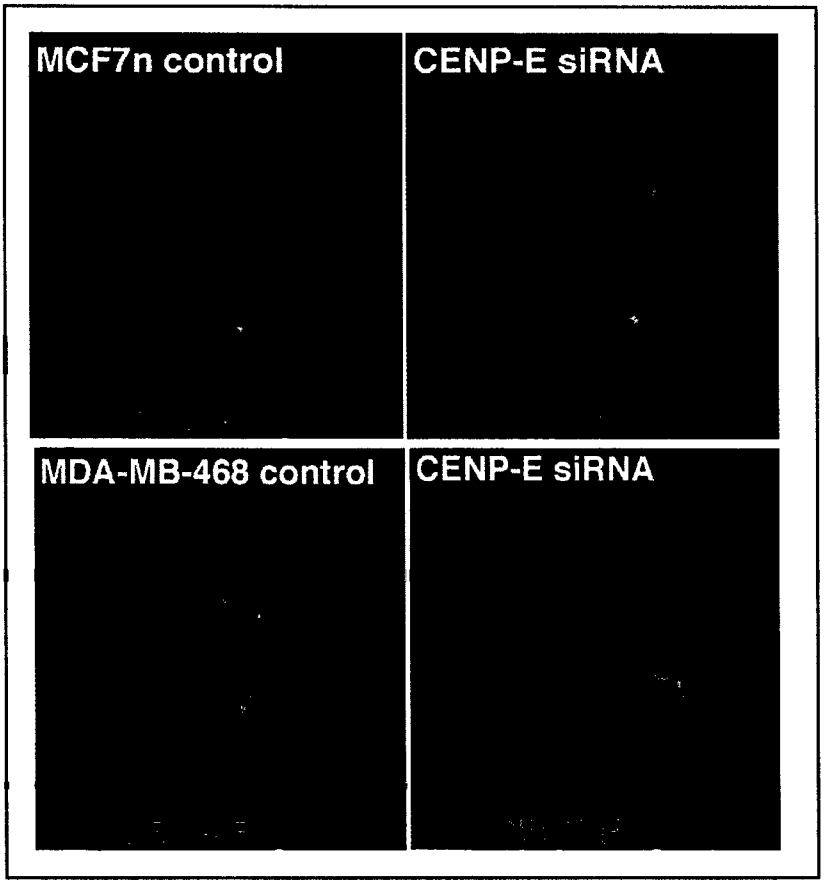

Figure 3. MDA468 and MCF cells were trans-fected with a control (left panels) or CENP-E (right panels) siRNAs and mitotic cells were stained with rabbit anti-CENP-E, mouse anti-dynein and DNA. Images were captured with a 63X oil objective and then pseudo-colored and merged.

Although not an official aim of this project, we recently obtained the crystal structure of the monomeric motor domain of CENP-E and the results of these studies are now in press. This work was conducted in collaboration with Dr. Kozielski who is a crystallographer. The purpose of this study is to use the structure to design chemical inhibitors of CENP-E. As we have previously shown that the motor domain of CENP-E is critical for its function in vivo, such inhibitors would complement the RNAi approach to knockdown CENP-E function. Inhibitors have been identified but they are not cell permeable. Ongoing efforts are to derivatize the compounds to improve cell uptake.

Task 4. Maintaining stocks of affinity purified antibodies.

Over the past year, we have generated monoclonal antibodies to hBUB1, hBUBR1 and MAD1 proteins. The existence of monoclonal antibodies to these and other checkpoint proteins provides us with a continuous source of high quality antibody. While the efforts to generate monoclonal antibodies are significant, we are certain that it will reduce the labor that is required to maintain stocks of polyclonal antibodies. We have recently generated a monoclonal antibody against CENP-E. We did not succeed to generate any Mad2 monoclonal antibodies. We will continue to rely on polyclonal antibodies for those proteins that we do not have monoclonal antibodies against.

Key Research Accomplishments:

- Confirmed expression and localization of CENP-E and the mitotic checkpoint proteins hBUB1, hBUBR1, MAD1, MAD2, Cdc20 and CENP-E, in MCF7 and MDA468 cells.
- Verified that siRNA can inhibit the expression of CENP-E and hBUB1 in MCF7 and MDA468 cells.
- Generated monoclonal antibodies to hBUB1, hBUBR1,MAD1 and CENP-E.
- Obtain the crystal structure of the CENP-E motor domain.

Reportable Outcomes:

Jablonski, S.A., Liu, S.T., Yen, T.J. Targeting the kinetochore for mitosis-specific inhibitors. *Cancer Biol. Ther.* **2**:21-26, 2003.

Joseph, J., Liu, S.-T., Jablonski, S.A., Yen, Tim J., Dasso, M. The RanGAP1-RanBP2 complex is essential for microtubule-kinetochore interactions in vivo. *Curr. Biol.* **14**:611-617, 2004.

Liu, S.T., van Deursen, J., Yen, T.J. The role of the mitotic checkpoint in maintaining genomic stability. *Curr. Top. Dev. Biol.* Edited by G. Schatten (in press).

Garcia-Saez, I., Yen, T.J., Wade,R.H., Kozielski, F. Crystal Structure of the Motor Domain of the Human kinetochore protein CENP-E. *J. Mol. Biol.* (in press).

Licensed hBUB1 and hBUBR1 monoclonal antibodies to BD Sciences. Immuquest, Novus Biologicals, Chemicon.

Patent pending for the atomic coordinates for the CENP-E motor domain.

Conclusions:

We validated the expression of candidate target genes in various breast cancer lines and have used siRNA to inhibit their expression in these cells. However, the low transfection efficiencies has prevented us from conductiong clonogenic survival experiments. Nevertheless, we are optimistic that the viral delivery system will allow populations of cells to be uniformly infected with recombinant lentivirus that express siRNA. This will allow us to reliably evaluate results from clonogenic studies.

The availability of the crystal structure of the motor domain of CENP-E will afford the opportunity to obtain chemical inhibitors that can be used to test for clonogenic assays. This in combination with siRNA will enhance our ability to target multiple mitotic proteins to assess their importance to the viability of breast cancer cells.

Appendices:

Jablonski, S.A., Liu, S.T., Yen, T.J. Targeting the kinetochore for mitosis-specific inhibitors. *Cancer Biol. Ther.* **2**:21-26, 2003.

Joseph, J., Liu, S.-T., Jablonski, S.A., Yen, Tim J., Dasso, M. The RanGAP1-RanBP2 complex is essential for microtubule-kinetochore interactions in vivo. *Curr. Biol.* **14**:611-617, 2004.

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TIM J. YEN, Ph.D., PRINCIPAL INVESTIGATOR

"Validate Mitotic Checkpoint and Kinetochore Motor Proteins in Breast Cancer Cells as Targets for the Development of Novel Anti-Mitotic Drugs"

Appendix

Jablonski, S.A., Liu, S.T., Yen, T.J. Targeting the kinetochore for mitosis-specific inhibitors. *Cancer Biol. Ther.* **2**:21-26, 2003.

Joseph, J., Liu, S.-T., Jablonski, S.A., Yen, Tim J., Dasso, M. The RanGAP1-RanBP2 complex is essential for microtubule-kinetochore interactions in vivo. *Curr. Biol.* **14**:611-617, 2004.

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Review

Targeting the Kinetochore for Mitosis-Specific Inhibitors

S.A. Jablonski
S.T. Liu
T.J. Yen*

Fox Chase Cancer Center; Philadelphia, Pennsylvania USA

*Correspondence to: Tim J. Yen; Fox Chase Cancer Center; 7701 Burholme Avenue; Philadelphia, Pennsylvania 19111 USA; Tel.: 215.728.2590; Fax: 215.728.2412; Email: tj_yen@fccc.edu

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mitosis, kinetochore, drug target

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ABSTRACT

Microtubule poisons such as taxol and vinblastine are widely used to treat a variety of cancers. These drugs are believed to kill cells by blocking mitosis. However, there is a critical need to identify new drugs because tumors can often become refractile to treatment with existing drugs. Studies over the past decade on chromosome segregation have uncovered a plethora of novel proteins that function specifically in mitosis. Centrosomes and kinetochores are two organelles that specify formation of the spindle and the attachment of chromosomes to the spindle, respectively. The focus of this review is to highlight the kinetochore as a rich source of targets for the development of mitosis-specific drugs.

INTRODUCTION

Microtubules are dynamic polymers that support a variety of cellular functions such as vesicle and organelle transport, cell shape and polarity, beating cilia, and spindle functions in mitosis. The latter function is the most desirable target for cancer chemotherapy. Indeed, the use of anti-microtubule drugs such as paclitaxel, docetaxel and the vinka alkaloids for the treatment of a variety of cancers is based on the ability of these drugs to inhibit mitosis and thus cell proliferation.[1] As these drugs inhibit other essential microtubule-based processes, they cannot specifically target mitotic cells. Of greater concern however is the high frequency at which cancer cells develop resistance to these drugs.[2] Cancer cells frequently become resistant as a result of elevated P-glycoproteins, alterations in the levels of tubulin isotypes or mutations in the tubulin subunits. For these reasons, the need to identify new drugs that inhibit the proliferation of cancer cells is imperative.

The kinetochore is a macromolecular complex that was first described at the EM level as a trilaminar disc that is situated on opposite sides of the highly condensed centromeric heterochromatin.[3-5] This structure is only visible in mitosis and therefore suggests that its assembly is a cell-cycle regulated event. For the purposes of this review, we define centromeres as the cis-acting DNA sequences that specify the assembly of a constellation of proteins that are considered to form the kinetochore (Table 1). Amongst these are proteins that specify microtubule attachments and checkpoint functions. Separately, there is a large number of proteins that are thought to play a structural role by contributing to the formation of a highly organized trilaminar kinetochore.[4,5] As many of these proteins are believed to function exclusively in mitosis, they are ideal candidates for drug discovery (Fig. 1).

KINETOCHORE MICROTUBULE ATTACHMENTS

One of the most important kinetochore functions in mitosis is establishing connections between the spindle and chromosomes.[6] Anti-microtubule drugs inhibit mitosis by interfering with the attachement of chromosomes to the spindle. However, direct inhibition of kinetochore proteins that are important for establishing microtubule connections exclusively in mitosis has the advantage of selectively targeting rapidly dividing cells. Kinetochores of vertebrate cells contain three known microtubule-based motors that include dynein, and the kinesin-like proteins, CENP-E and MCAK.[7-10] In addition, microtubule binding proteins such as CLIP170, EB1, and CLASP/Orbit are also present at kinetochores where they are also likely to mediate microtubule interactions with the kinetochore.[11-13] Given that the molecular motors are ATPases, they are better suited for drug development as it is possible to use the existing libraries of ATP analogs to screen for a suitable inhibitor. As dynein function is required in a wide variety of cellular processes besides mitosis, compounds that directly inhibit dynein will not achieve the desired specificity. On the other hand, CENP-E and MCAK are suitable candidates because they

appear to be only critical for mitosis. Cells depleted of CENP-E fail to align their chromosomes properly and arrest in mitosis despite the formation of a normal looking bipolar spindle.[14,15] As the motor domain of CENP-E has been shown to be essential for its function, it may be possible to identify inhibitors by screening for compounds that inhibit its ATPase activity. Although studies to examine the effect of disrupting CENP-E on cell killing remain unknown, it is clearly an essential gene in mouse.[16] Given its importance in mitosis, it is not surprising that CENP-E knockout mice die during early stages of embryogenesis. These observations strongly suggest that disruption of CENP-E will cause cells to die.

MCAK is an unconventional kinesin because it does not behave as a classic motor that translocates along the microtubule lattice. Instead, MCAK and its frog homolog, XKCM1, induce microtubule shortening.[17,18] At kinetochores, MCAK is believed to stimulate the depolymerization of the attached microtubules so that chromosome can move towards the poles. Thus, cells disrupted of MCAK accumulate lagging chromosomes in anaphase that is consistent with the biochemical properties of this protein. The presence of lagging chromosomes implies that these cells will divide and become aneuploid. One likely outcome is that many of the aneuploid progeny cells die.

TARGETING THE MITOTIC CHECKPOINT

The mitotic checkpoint is an evolutionarily conserved mechanism that prevents cells with even a single unaligned chromosome from exiting mitosis.[19] Cells with unaligned chromosomes that override the checkpoint will divide and produce aneuploid cells. Given that the majority of aneuploid cells will die because of massive chromosome imbalance,[20,21] the mitotic checkpoint should in principle be a reasonable target for drug development. It is clear that disruption of kinetochore proteins such as CENP-E or the spindle with conventional anti-microtubule drugs will arrest cells in mitosis. What is less clear is how these cells eventually die. One possibility is that cells die as a

Table 1 **CENTROMERE/KINETOCHORE PROTEINS IN HUMAN AND SEVERAL MAJOR MODEL ORGANISMS**

The known human centromere/kinetochore proteins are classified into seven categories. Not all the centromere/kinetochore proteins in model organisms are shown here. Different names of the same protein are separated by slash (/). Different proteins are separated by comma (,). The classification is not very strict, and some proteins have been put into more than one category. The references on individual proteins can be provided on request.

(A) Constitutive Centromere Proteins—Localized at Centromeres Throughout the Cell Cycle

Human	*S. cerevisiae*	*S. pombe*	*Drosophila*	*C. elegans*	*Xenopus*
CENP-A	Cse4	CENP-A/Cnd1	Cid	HCP3	CENP-A
CENP-B	Cbf1	Abp1, Cbh1, Cbh2			
CENP-C	Mif2			HCP4	
CENP-D					
CENP-G					
CENP-H		Sim4			
CENP-I/LRPR1	Ctf3	Mis6			
hMIS12	Mtw1	Mis12			

(B) Regulatory Proteins—Mitotic Checkpoint Proteins and Other Proteins Possibly Involved in the Mitosis Regulation

Human	*S. cerevisiae*	*S. pombe*	*Drosophila*	*C. elegans*	*Xenopus*
MAD1	Mad1	Mad1		MDF1	xMAD1
MAD2	Mad2	Mad2		MDF2	xMAD2
BUBR1	Mad3,Bub1	Mad3			xBUBR1
BUB1	Bub1	Bub1	BUB1	CeBUB1	xBUB1
BUB3	Bub3	Bub3	BUB3		xBUB3
MPS1/TTK	Mps1	Mph1			xMPS1
hZW10			ZW10	CeZW10	ZW10
hROD			ROD	Ce ROD	ROD
RAE1					
3F3/2 mAb antigens			3F3/2 mAb antigens		3F3/2 mAb antigens
CDC20/p55	Cdc20	Slp1	Fizzy/Fzy	FZY1/Fizzy	CDC20
APC1	Apc1	Cut4		MAT-2	APC1/BimE
APC3/ CDC27	Cdc27	Nuc2		MAT-1	APC3/CDC27
APC10/DOC1	Apc10/Doc1	Apc10			APC10
PLK1	Plk1/Cdc5	Plo1	POLO		Plx1
ERK					
PP1γ					

(C) Microtubule Motors or Associated Proteins Whose Kinetochore Localization is Independent of Mictrotubules

Human	*S. cerevisiae*	*S. pombe*	*Drosophila*	*C. elegans*	*Xenopus*
CENP-E			CENP-meta, CENP-ana		CENP-E
Dynein/dynactin			dynein		
MCAK				CeMCAK	XKCM1
hZW10			ZW10	CeZw10	xZW10
hROD			ROD	CeROD	xRod
hZWint-1			Zwint-1		
hZWilch			Zwilch		
CLIP-170	Bik1	Tip1	D-CLIP-190		
CLASP	Stu1		MAST/Orbit		
HEC1	Ndc80	Ndc80			xNDC80
hNuf2	Nuf2	Nuf2		HIM10	xNUF2

(D) Microtubule Associated Proteins Whose Kinetochore Localization is Dependent Upon Mictrotubules

Human	*S. cerevisiae*	*S. pombe*	*Drosophila*	*C. elegans*	*Xenopus*
LIS1	Pac1				
APC					
EB1	Bim1	Mal3			
ch-TOG	Stu2	Dis1, Mtc1/Alp4			XMAP215

Table continued on next page.

Table 1 **CENTROMERE/KINETOCHORE PROTEINS IN HUMAN AND SEVERAL MAJOR MODEL ORGANISMS, CONT.**

The known human centromere/kinetochore proteins are classified into seven categories. Not all the centromere/kinetochore proteins in model organisms are shown here. Different names of the same protein are separated by slash (/). Different proteins are separated by comma (,). The classification is not very strict, and some proteins have been put into more than one category. The references on individual proteins can be provided on request.

(E) Chromosomal passenger Proteins

Human	S. cerevisiae	S. pombe	Drosophila	C. elegans	Xenopus
Aurora-B/ AIRK2	Ipl1	Ark1	Aurora B	AIR-2	AuroraB
Survivin	Bir1	Cut17/Bir1	survivin	ICP-1, ICP-2?	survivin
INCENP	Sli15	Pic1	INCENP	INCENP	INCENP
TD6					

(F) Nuclear Pore Proteins

Human	S. cerevisiae	S. pombe	Drosophila	C. elegans	Xenopus
RanGAP					
Nup358/RanBP2					
Nup133					
Nup105					
MAD1	MAD1				xMAD1
MAD2	MAD2				xMAD2
MPS1/TTK					

(G) Structural Proteins/Unknown Functions

Human	S. cerevisiae	S. pombe	Drosophila	C. elegans	Xenopus
CENP-F				HCP1, HCP2	
53BP1					53BP1
Topolla					
PARP1					
cohesin					
hRAD21/hSCC1	Mcd1/Scc1/Rad21	Rad21			xSCC1
STAG1/SA2	Scc3	Psc3			
STAG2/SA2	Scc3				
STAG3	Scc3				
hSMC1	Smc1	Psm1/Smc1			xSMC1
hSMC3	Smc3	Psm3/Smc3			xSMC3
hPDS5	Pds5	Pds5			
condensin					
hCAP-E/SMC2	Smc2	Cut14		MIX1	xCAP-E
hCAP-C/SMC4	Smc4	Cut3		SMC1	xCAP-C
hCAP-H/BRRN	Brn1	Cnd3	BARREN		xCAP-H/ Barren
hCAP-G	Ycs4	Cnd2			xCAP-G
hCAP-D2/ CNAP1/hEg7		Cnd1			xCAP-D2/ pEg7

result of overriding the checkpoint. The biochemical activities of checkpoint proteins that arrest cells in mitosis cannot be sustained indefinitely. Thus, checkpoint arrested cells eventually will exit mitosis regardless of whether their chromosomes have properly aligned or not. Cells that exit with unaligned chromosomes will become aneuploid and die because they lack chromosomes that are essential for life. Inhibition of the mitotic checkpoint may also be used in conjunction with conventional anti-microtubule drugs to enhance cell killing. One of the responses of tumors when they are exposed to microtubule poisons is to activate the mitotic checkpoint. If tumor cells are able to mount a robust checkpoint response, they may remain blocked in mitosis until the drug is metabolized, at which time they resume progression through the cell cycle. While this response may be difficult to document in patients, it is feasible to test whether inhibition of the mitotic checkpoint will sensitize cells in culture to existing microtubule poisons.

The search for inhibitors of the mitotic checkpoint is now possible because many proteins that are essential for this process have been identified. Pioneering studies in budding yeast identified six evolutionarily conserved proteins, Mad1, Mad2 Mad3, Bub1, Bub3 and Mps1, that are essential for cells to arrest in mitosis in the presence of spindle damage.[22-24] In metazoans, many of these proteins have been shown to localize to kinetochores where they are postulated to monitor the status of microtubule attachments and the amount of kinetochore tension that develops as a consequence of opposing poleward forces.[19] Although the precise role of each of these proteins in the checkpoint pathway remains to be clarified, it is clear that all of them are essential for cells to arrest in mitosis in response to unattached kinetochores. Given that MPS1, BUB1 and the Mad3-related BUBR1 are all protein kinases, it is likely that they are part of a kinase cascade that initiates at an unattached kinetochore and is amplified throughout the cell to inhibit the ubiquitin ligase Anaphase Promoting Complex. These kinases are therefore prime targets for drug development given the existence of large libraries of compounds that were designed for identifying kinase inhibitors. In contrast to the protein kinases, the biochemical properties of the Bub3, Mad1 and Mad2 checkpoint proteins remain unknown as their primary sequence do not reveal distinctive motifs. As these proteins are known to form complexes with each other and some of the checkpoint kinases, it may be possible to screen for compounds that disrupt these protein interactions. This is a feasible strategy given that overexpression of a specific fragment of Mad1 will sequester the endogenous Mad2 so that it cannot form complexes with other checkpoint proteins in the cell.[25] As a consequence, cells overexpressing the Mad1 fragment are unable to arrest in mitosis.

IDENTIFICATION OF A KINETOCHORE ASSEMBLY PATHWAY

The observation that kinetochores are only visible in mitosis suggests that this structure undergoes cell cycle dependent assembly and disassembly. This is supported by the existence of proteins that are only detected at kinetochores in mitosis. Kinetochore proteins can be categorized as constitutive or transient based on their temporal patterns of localization in human cells. Proteins such as CENP-A, CENP-B, CENP-C, CENP-G, CENP-H, CENP-I and hMIS12 belong to the constitutive class because they are localized to kinetochores throughout the cell cycle (strictly speaking, they are associat-

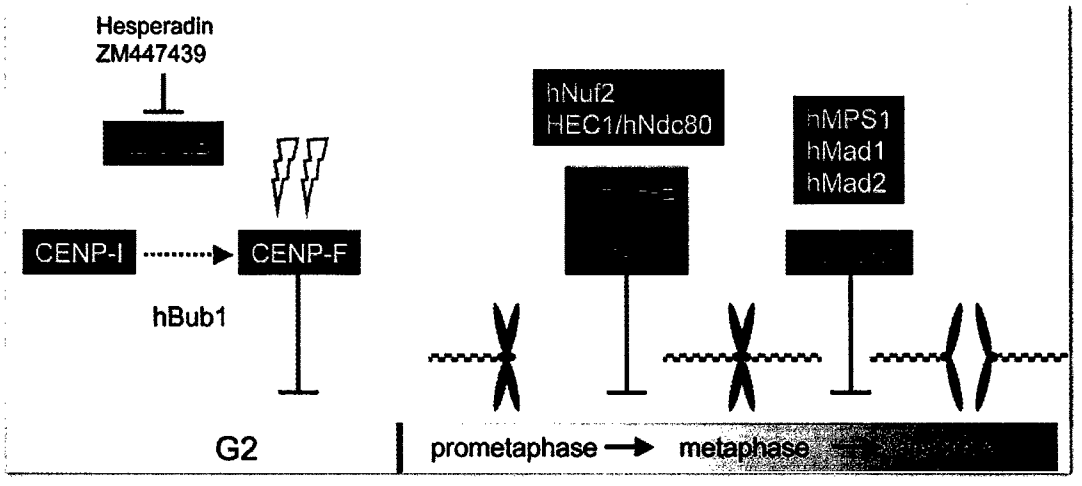

Figure 1. Schematic diagram of the kinetochore assembly pathway and potential sites for drug intervention. Kinetochore proteins are depicted according to their approximate temporal pattern of localization. Proteins that lie along the same assembly pathway are depicted in the same color. The exception is that the localization of Mad1 and Mad2 are known to be dependent on CENP-I and HEC1/hNdc80 but it is unknown whether these proteins define two converging pathways or lie along the same pathway. Critical transition points in the cell cycle are defined by CENP-F, the microtubule motors, and checkpoint proteins. These sites may be targets for drugs or radiation.

ed with pre-kinetochores during interphase). A larger group of proteins are only transiently detected at kinetochores during mitosis (Table 1). Thus, the constitutive kinetochore proteins form a core to which transient kinetochore proteins are recruited to in a cell-cycle dependent manner. For example, the kinesin-like motor protein, CENP-E, accumulates in the cytoplasm during interphase and is not detected at kinetochores until the onset of mitosis.[26] Interestingly, not all transient kinetochore proteins are detected at kinetochores at the same time. A careful comparison of the temporal localization pattern of various members of the transient family of kinetochore proteins revealed a distinct order of assembly (Fig. 1). For example, CENP-F is a protein that is uniformly distributed in nuclei of cells in the G_2 phase of the cell cycle. It is first detected at kinetochores during late G_2 and thus precedes the appearance of CENP-E at kinetochores.[27] Similarly, the hBUB1 checkpoint kinase is also first detected at kinetochores in G_2. Remarkably, its appearance at kinetochores precedes CENP-F.[28] These observations suggest that proteins such as hBUB1 and CENP-F may define discrete segments of G_2 and that this phase of the cell cycle may specify kinetochore assembly much like DNA replication defines S phase.

The recent characterization of human CENP-I has revealed some additional complexities to the kinetochore assembly process.[29] CENP-I is a constitutive kinetochore protein that exhibits limited sequence similarity with Mis6 and Ctf3 kinetochore proteins in fission and budding yeast, respectively. Unlike Mis6 which is essential for the loading of the histone H3 variant, CENP-A, onto centromeres in fission yeast,[30] this relationship does not appear to be conserved in humans, chickens and budding yeast.[29,31,32] In Hela cells. CENP-I was shown to be essential for CENP-F, Mad1 and Mad2 to assemble onto kinetochores. The failure of these proteins to assemble onto kinetochores is not due to gross disruption of this structure as other transient kinetochore proteins such as CENP-E, dynein, hBUB1 and hBUBR1 were not affected by the loss of CENP-I. These data show that kinetochore assembly does not follow a single linear pathway but is branched. Each branch may specify a discrete functional domain within the kinetochore.

Studies have also revealed that the kinetochore assembly pathway maybe monitored by the checkpoint. Hela cells depleted of CENP-I were found to be delayed in G_2 at a stage when CENP-F was still distributed uniformly in the nucleus.[29] This delay was estimated to last for approximately three hours before cells resumed their progression into mitosis. It is unknown at this time how kinetochore assembly is monitored. Interestingly, kinetochore assembly appears to be linked to the DNA damage checkpoint. Hela cells that were exposed to ionizing radiation or etoposide were found to accumulate at a discrete stage in G_2 prior to when CENP-F assembled onto kinetochores.[33] The molecular basis for this connection is not clear but it is likely that the DNA damage response inhibits key cell-cycle regulators whose activities are required for CENP-F to assemble onto the growing kinetochore. It is perhaps not a coincidence that cells depleted of CENP-I are delayed at the same point in G_2 as the cells with DNA damage. The combined results strongly suggests that the assembly of CENP-F onto kinetochores might represent a critical point in G_2 where it is subject to checkpoint intervention. It is possible that the assembly of CENP-F onto kinetochores may represent a key transition point where cells commit themselves to proceed into the next phase of the cell cycle.

DISRUPTING KINETOCHORE ASSEMBLY

We believe that the ordered assembly of proteins onto kinetochores may serve two purposes (Fig. 1). The first is to provide a mechanism to ensure that a kinetochore is properly assembled. Thus, the successful assembly of one protein may be critical for the subsequent assembly of proteins onto the growing kinetochore. The second reason is that the order of assembly reflects the spatial organization of discrete functional domains to kinetochores. Thus, the disruption of kinetochore assembly may be a strategy that effectively inhibits multiple proteins that provide critical functions to kinetochores. This approach may be an important alternative to conventional approaches to search for inhibitors of kinases or molecular motors. In those cases, it may be difficult to identify inhibitors with

the desired specificity. This is a serious concern as there are a large number of protein kinases and kinesin-like proteins that provide critical functions to non-dividing cells. By contrast inhibitors of the putative structural proteins may be more specific as they must act by disrupting interactions with other proteins.

Recent reports of the human kinetochore proteins hNuf2, HEC1/hNdc80 help to illustrate the utility of inhibiting kinetochore assembly.[34,35] Nuf2 and Ndc80 were originally identified in budding yeast as part of a tetrameric complex that includes Spc25 and Spc25.[36] In human cells, both hNuf2 and HEC1/hNdc80 localize to kinetochores consistent with their yeast counterparts. Hela cells depleted of hNuf2 and HEC1/hNdc80 accumulate in mitosis because their chromosomes failed to attach to the spindle.[34,35] Kinetochores in cells depleted of HEC1/hNdc80 lack detectable levels of the checkpoint proteins Mad1, Mad2, Mps1 and reduced amounts of hBUB1.[34] Similarly, kinetochores depleted of hNuf2 also show reduced levels of Mad2 and hBubR1[35] (Jablonski and Yen, unpublished data). Although the long-term fate of the HEC1/hNdc80 depleted cells is not known, the hNuf2 depleted cells arrest in mitosis but eventually die[35] (Jablonski and Yen, unpublished data). Kinetochores lacking hNuf2 or HEC1/hNdc80 retained CENP-E and dynein but the localization of other microtubule binding proteins is not known. As hNuf2 and HEC1/hNdc80 are not known to interact directly with microtubules, the mechanism by which they specify attachment of chromosomes to the spindle is unclear. Regardless of how loss of these proteins disrupt chromosome segregation, the outcome of inhibiting of these proteins is very similar to that when dividing cells are treated with microtubule poisons.

A significant obstacle in searching for inhibitors to proteins such as hNuf2 and HEC1/hNdc80 is that it is difficult to develop in vitro assays without the knowledge of their biochemical activities. However, it may be possible to take advantage of the fact that these proteins and the pathway they specify are conserved in yeast. The strategy to use yeast as a tool to identify drugs that target pathways that are conserved between yeast and humans was developed a number of years ago as part of the NCI sponsored Seattle Project.[37] As yeast grow rapidly, compounds can be simply assayed by monitoring their effects on growth. As the assay can be conducted in microtiter plates, it facilitates automation and large scale screening efforts. As HEC1/hNdc80 has been reported to complement ndc80 mutant yeast, a screen could in principle be designed to identify inhibitors of the human homolog.

INHIBITORS OF KINETOCHORE PROTEINS

Farnesyl transferase inhibitors (FTI) which were designed to inhibit cancer cells that express constitutively active Ras mutants have since been found to interfere with mitosis in some cell lines.[38-40] It is interesting that both CENP-E and CENP-F are farnesylated and thus raise the possibility that they may be targets for inhibition by FTI's.[38] However, two studies using different FTI's (FTI-2153 and SCH66336) showed that the localization of both proteins to kinetochores was unaffected by these drugs.[38,39] Nevertheless, mutation of the invariant C in the CAAX motif prevented CENP-F from accumulating at kinetochores.[40] These results suggest that the localization of CENP-F and possibly CENP-E is dependent on farnesylation. In the presence of FTI's, CENP-E and CENP-F may undergo alternate modifications such as geranylgeranylations that may be sufficient for their localization to kinetochores. Regardless of the effects of FTI's on CENP-E and CENP-F. the FTI-253 was shown to block the lung cancer cell lines A-549 and Calu-1 arrest in mitosis.[39] The arrest was attributed to the failure to establish a bipolar spindle. This observation suggests that FTI's may have additional targets in the centrosome.

Aurora kinase plays a critical role in mitosis as it is important for spindle formation and attachment of chromosomes to the spindle.[41] Mammalian cells express three versions of aurora. Aurora A and B are localized to centrosomes and kinetochores respectively. Aurora C expression appears to be restricted to testes so its role in mitosis is not certain. The Aurora kinases were targeted for drug discovery as their expression was elevated in many cancer cell lines.[42] Two recent studies report on the characterization of inhibitors of aurora kinase in mammalian cells.[43,44] Hesparadin and ZM447439 are kinase inhibitors that appear to be fairly specific for aurora kinases in vitro. Although both inhibitors are equally effective in inhibiting Aurora A and B in vitro, their effects on cells suggest Aurora B maybe more sensitive in vivo. Aurora B is localized to kinetochores where it is believed to facilitate attachment of microtubules.[45] In yeast, the aurora-related, Ipl1 kinase is believed to ensure that kinetochores do not become attached to the same pole.[46] Such "syntelic" attachments would escape detection by the checkpoint because the kinetochores are saturated with microtubules. If uncorrected, chromosomes would remain attached to a single pole and undergo non-disjunction. Ipl1 is thought to be able to detect monopolar attached chromosomes because their kinetochores do not develop sufficient tension. Ipl1 is therefore thought to stimulate the release of microtubules from kinetochores that are not under tension. This has important implications for the anti-cancer drug taxol as this drug is known to suppress microtubule dynamics and thus prevent the establishment of tension at kinetochores. The absence of tension is believed to be the mechanism by which taxol treated cells remain arrested in mitosis despite the fact that they have a fully formed spindle that is attached to chromosomes. This contrasts with drugs such as vinblastine and colchicine which arrest cells in mitosis because kinetochores lack microtubule attachments. Remarkably, inhibition of Aurora B with Hesparadin and ZM447439 abrogated the taxol mediated arrest and caused cells to exit. In contrast, their effects are much lower in affeting the ability of microtubule destabilizing drugs such as nocodazole from arresting cells in mitosis.[43,44] These results suggest that inhibition of Aurora B might sensitize tumor cells to treatment with taxol but not the vincalkaloids.

CONCLUSIONS

Studies over the past decade have revealed a highly conserved mechanism by which eucaryotic cells accurately segregate their chromosomes. These studies have identified many molecular components that are essential for this process. As such, these proteins should be ideal candidates for the development of highly specific anti-mitotic drugs for the treatment of cancer (Fig. 1). Although this review has focused on kinetochores, there are many proteins that are involved in spindle assembly that also qualify as targets for drug discovery. Thus, the future challenge is no longer target identification but target selection.

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The RanGAP1-RanBP2 Complex Is Essential for Microtubule-Kinetochore Interactions In Vivo

Jomon Joseph,[1] Song-Tao Liu,[2]
Sandra A. Jablonski,[2] Tim J. Yen,[2] and Mary Dasso[1,*]
[1]Laboratory of Gene Regulation and Development
National Institutes of Health
National Institute of Child Health
and Human Development
Building 18, Room 106
18 Library Drive, MSC-5431
Bethesda, Maryland 20892
[2]Institute for Cancer Research
The Fox Chase Cancer Center
333 Cottman Avenue
Philadelphia, Pennsylvania 19111

Summary

RanGAP1 is the activating protein for the Ran GTPase. Vertebrate RanGAP1 is conjugated to a small ubiquitin-like protein, SUMO-1 [1, 2]. This modification promotes association of RanGAP1 with the interphase nuclear pore complex (NPC) through binding to the nucleoporin RanBP2, also known as Nup358. During mitosis, RanGAP1 is concentrated at kinetochores in a microtubule- (MT) and SUMO-1-dependent fashion [3]. RanBP2 is also abundantly found on kinetochores in mitosis [3]. Here we show that ablation of proteins required for MT-kinetochore attachment (Hec1/Ndc80, Nuf2 [4–6]) disrupts RanGAP1 and RanBP2 targeting to kinetochores. No similar disruption was observed after ablation of proteins nonessential for MT-kinetochore interactions (CENP-I, Bub1, CENP-E [7–9]). Acquisition of RanGAP1 and RanBP2 by kinetochores is temporally correlated in untreated cells with MT attachment. These patterns of accumulation suggest a loading mechanism wherein the RanGAP1-RanBP2 complex may be transferred along the MT onto the kinetochore. Depletion of RanBP2 caused mislocalization of RanGAP1, Mad1, Mad2, CENP-E, and CENP-F, as well as loss of cold-stable kinetochore-MT interactions and accumulation of mitotic cells with multipolar spindles and unaligned chromosomes. Taken together, our observations indicate that RanBP2 and RanGAP1 are targeted as a single complex that is both regulated by and essential for stable kinetochore-MT association.

Results and Discussion

We previously reported that RanGAP1 associates with kinetochores in a SUMO-1- and MT-dependent fashion [3]. Several lines of evidence indicated that this targeting occurs in association with RanBP2. First, these proteins remain tightly bound throughout the cell cycle. Anti-RanBP2 antibodies precipitated SUMO-1-conjugated RanGAP1 with equal efficiency from interphase and mitotic HeLa cell extracts (Figure S1A). Equal coprecipitation efficiencies were also observed between interphase and mitotic *Xenopus* egg extracts (data not shown). Second, RanGAP1 and RanBP2 colocalize during mitosis [3]. Third, RanGAP1 spindle localization is absolutely dependent upon RanBP2 (Figure S1B). Moreover, RanBP2 and RanGAP1 recruitment to kinetochores showed identical requirements for other kinetochore components and identical timing during unperturbed cell cycles (see below). These results provide strong support for our earlier speculation that RanGAP1 and RanBP2 are targeted as a complex during mitosis [3]. For the purposes of this report we have thus assumed that RanGAP1 and RanBP2 are localized on spindles as a single entity.

We have investigated how and why the RanGAP1-RanBP2 complex is targeted to kinetochores. We initially examined which features of the kinetochore are critical for recruitment of RanGAP1 and RanBP2. Because their kinetochore localization was MT dependent, we utilized RNAi to suppress the expression of kinetochore proteins that are required for stable MT-kinetochore interactions (Hec1/Ndc80 and Nuf2 [4–6]) and analyzed the effect on RanGAP1 and RanBP2 targeting. We also examined the localization of RanGAP1 and RanBP2 after the depletion of CENP-E. CENP-E loss does not abolish MT attachment but does cause decreased numbers of MT-kinetochore attachments and loss of tension on kinetochores [9, 10]. Finally, we examined the fate of RanGAP1 and RanBP2 after depletion of CENP-I and Bub1 [7, 8], kinetochore proteins that are implicated in other aspects of kinetochore function and in spindle checkpoint signaling.

Depletion of Hec1 and Nuf2 by RNAi compromised kinetochore-MT attachment in mitotic cells and thus impaired chromosome alignment (Figure 1) [6, 11]. Under these circumstances, RanGAP1 and RanBP2 were no longer associated with kinetochores, showing that Hec1 and Nuf2 are essential for targeting both proteins (Figure 1, data not shown). While Hec1 and Nuf2 are required for recruitment of the RanGAP1-RanBP2 complex, they are not sufficient: Hec1 and Nuf2 are retained on kinetochores in nocodazole-treated cells ([5]; data not shown), although RanGAP1 and RanBP2 are not [3]. This fact suggests either that Hec1 and Nuf2 are not competent to recruit the RanGAP1-RanBP2 complex prior to MT-kinetochore attachment or that they are required indirectly through their role in stabilizing MT interactions. We favor the latter alternative, since there is no precedent for direct interaction of Hec1 or Nuf2 with the RanGAP1-RanBP2 complex. Consistent with this notion, RNAi-mediated depletion of CENP-I and Bub1, which are not essential for MT-kinetochore attachment [7, 8], had no effect on the kinetochore binding of either RanGAP1 (Figure S2A) or RanBP2 (data not shown).

As reported earlier [9, 10], depletion of CENP-E resulted in mitotic arrest with a mixture of aligned and unaligned chromosomes. In CENP-E-depleted cells,

*Correspondence: mdasso@helix.nih.gov

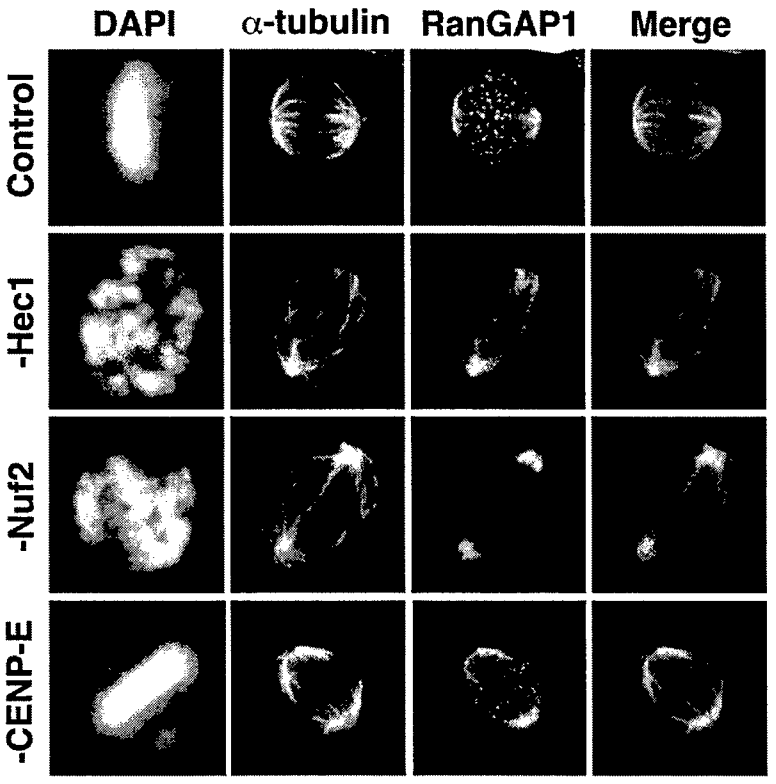

Figure 1. Hec1 and Nuf2 Are Required for RanGAP1-RanBP2 Complex Localization

HeLa cells were transfected with siRNA oligonucleotides specific for Hec1, Nuf2, and CENP-E for 24–48 hr to knockdown the expression of indicated proteins. Depletion of Hec1, Nuf2, and CENP-E could be achieved after 48 hr, as evidenced by the undetectable levels of these proteins at kinetochores in cognate RNAi-treated cells as compared to control RNAi-treated cells (data not shown). Cells were fixed as described in the Supplemental Experimental Procedures. Kinetochore localization of RanGAP1 (red) was examined by using specific antibodies. The microtubules were visualized with anti-α-tubulin (green) and DNA with DAPI (blue).

RanGAP1 and RanBP2 were found at kinetochores of aligned chromosomes, whereas kinetochores of unattached chromosomes lacked detectable staining (Figure 1, data not shown). In the absence of CENP-E function, MT-kinetochore attachment is achieved, although the number of kinetochore MTs is decreased and tension on kinetochores is compromised [9]. Notably, neither RanGAP1 nor RanBP2 was displaced as a result of decreased kinetochore tension after loss of CENP-E. Consistent with this observation, RanGAP1 and RanBP2 localized to kinetochores in taxol-treated cells (data not shown). Together, these data indicate that RanGAP1 and RanBP2 accumulation at kinetochores is compromised in the absence of MT attachment but does not appear to be sensitive to loss of kinetochore tension.

We further examined the correlation between MT attachment and RanGAP1-RanBP2 complex acquisition by kinetochores under unperturbed conditions through careful comparison of Mad1 and RanGAP1 localization in untreated HeLa cells. Mad1 and Mad2 are checkpoint proteins that become abundantly associated with unattached kinetochores in close correlation with spindle checkpoint activation [12]. As reported earlier [13], Mad1 (Figure 2A) and Mad2 (data not shown) localized on the nucleoplasmic side of the NPC during interphase; RanGAP1 (Figure 2A) and RanBP2 (data not shown) associated with the cytoplasmic side of the NPC [14]. During nuclear envelope (NE) breakdown in prophase, Mad1 prominently localized to kinetochores, although residual Mad1 could still be observed on the NE (Figure 2B). RanGAP1 (Figure 2B) and RanBP2 (data not shown) were still found at the NE at this stage, but not on kinetochores. After NE breakdown was complete, the RanGAP1-RanBP2 complex did not accumulate on kinetochores prior to MT attachment. This could be clearly seen through the mutually exclusive localization of RanGAP1 and Mad1 (Figure 2C) [12]. Mad1 localized to both kinetochores on a fully unattached chromosome, while RanGAP1 bound neither (Figure 2C, insert 1). The mutually exclusive localization of RanGAP1 and Mad1 was even more apparent in the case of a chromosome that had a single MT-attached sister kinetochore (Figure 2C, insert 2): Mad1 was found only on the unattached sister kinetochore, whereas RanGAP1 was found only on the attached sister. Like Mad1, Mad2 showed a distribution pattern that was inverse to the RanGAP1 pattern (data not shown). Furthermore, RanBP2 deposition on kinetochores was also mutually exclusive to both Mad1 and Mad2 accumulation (data not shown).

These data support the idea that RanGAP1-RanBP2 complex accretion on kinetochores is closely coupled to MT attachment under unperturbed conditions. It is plausible that MT attachment causes structural changes at the kinetochore that permit stable association of the RanGAP1-RanBP2 complex. Another attractive possibility that is not mutually exclusive with structural changes at kinetochores is that the RanGAP1-RanBP2 complex binds to MT and is transferred from the MT to the kinetochore. A similar loading model has previously been proposed for the DASH complex in budding yeast, which accumulates on maturing kinetochores in a MT-dependent fashion [15]. It is notable that the complementary kinetochore localizations of proteins from the nucleoplasmic (Mad1 and Mad2) and cytoplasmic (RanGAP1 and RanBP2) faces of the NPC are oppositely determined by MT attachment. Interestingly, the Nup107-

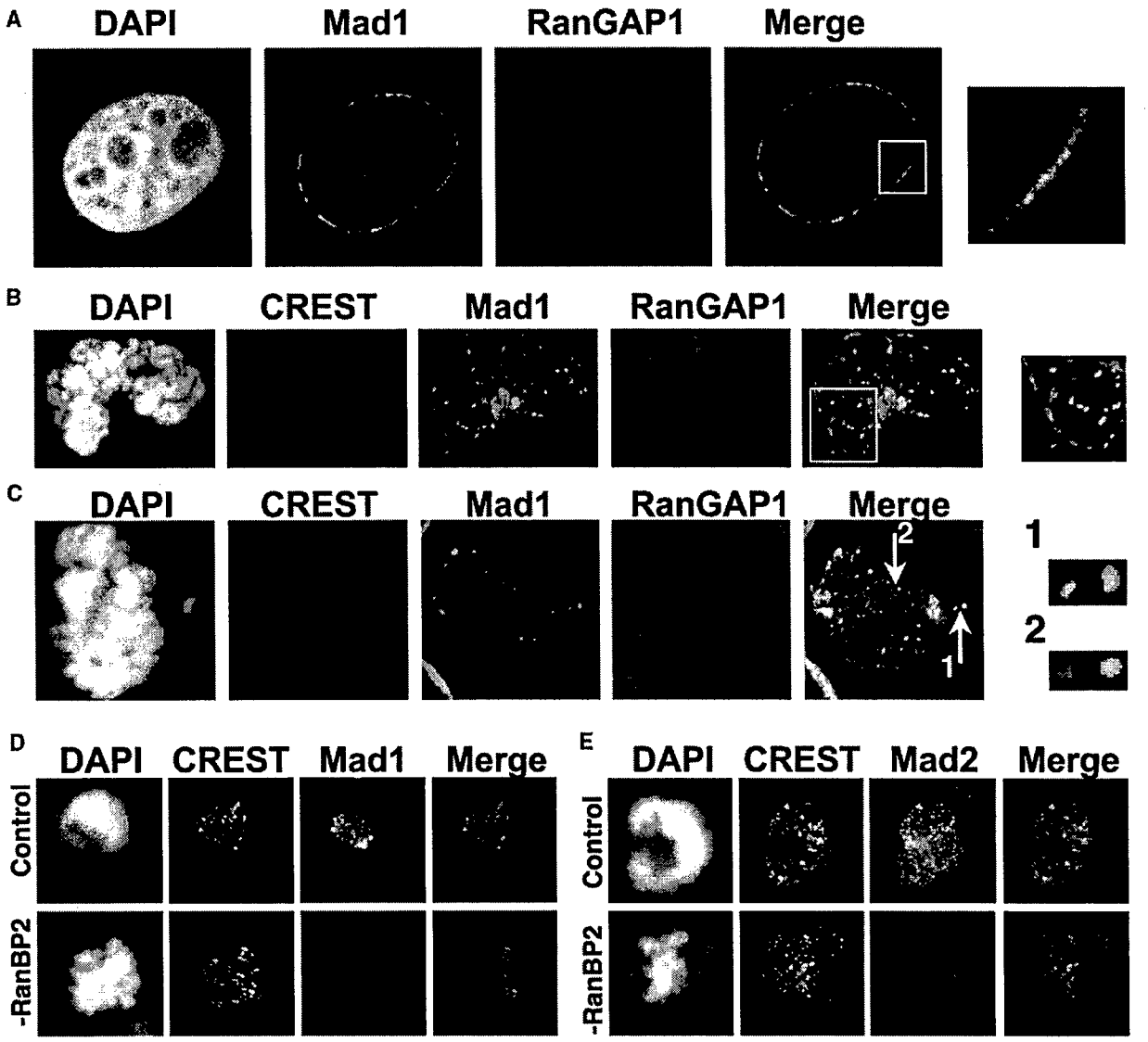

Figure 2. Mad1 and Mad2 Require the RanGAP1-RanBP2 Complex for Kinetochore Binding but Do Not Colocalize on Unattached Kinetochores

HeLa cells (A–C) or RGG cells (D and E) were permeabilized and fixed as described in the Supplemental Experimental Procedures. The cells in (A), (B), and (C) were stained for Mad1 (green) and RanGAP1 (red) with specific antibodies and fluorescent secondary antibodies. Where indicated, immunofluorescent staining with CREST sera is shown in blue to show the localization of mitotic centromeres. The cells in (D) and (E) were stained for CREST (Green) and Mad1 or Mad 2 (Red), as indicated.

(A) During interphase, RanGAP1 is localized on the cytoplasmic face of the NPC, while Mad1 staining is on the nucleoplasmic face of the NPC.

(B) During prophase, Mad1 is recruited to loci adjacent to centromeres prior to the release of RanGAP1 from the NPC.

(C) During metaphase, RanGAP1 and Mad1 show inverse staining patterns. Insert 1 shows a chromosome where both kinetochores are unattached; note the lack of RanGAP1 staining. Insert 2 shows a chromosome where a single kinetochore has become attached. Note that this kinetochore has both released Mad1 and acquired RanGAP1, while its sister remains associated exclusively with Mad1.

(D and E) RGG cells were transfected with RanBP2 siRNA oligonucleotides and examined by immunflourescence 86 hr after transfection. Note the absence of Mad1 and Mad2 recruitment during prophase to unattached kinetochores.

160 subcomplex of NPC proteins has been shown to reside on both sides of the pore during interphase [16], and it is bound to kinetochores in a MT-independent fashion from prophase to late anaphase [16]. It is attractive to speculate that the Nup107-160 subcomplex might play an important role in mitotic recruitment of proteins from both nuclear and cytoplasmic sides of the NPC to kinetochores. If this were the case, biochemical and/or structural changes in the Nup107-160 subcomplex in response to MT attachment may have some role in determining how other NPC components accumulate on the kinetochore.

To determine the significance of mitotic RanGAP1-RanBP2 complex targeting, we depleted RanBP2 by using RNAi in RGG cells, a stable, HeLa-derived cell line expressing a green fluorescent protein-labeled chimeric protein consisting of HIV-1 Rev and a hormone-inducible nuclear localization sequence (Rev-GR-GFP [17]). West-

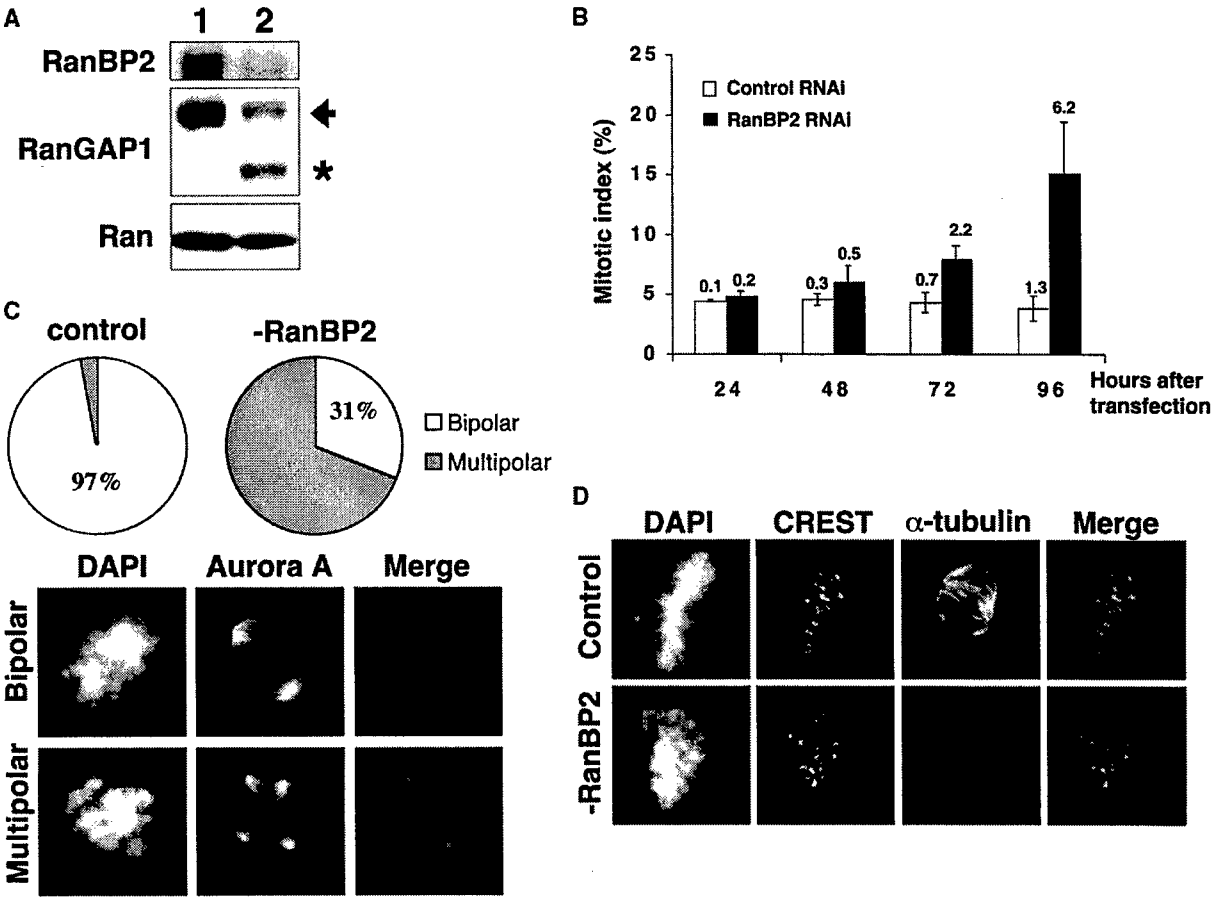

Figure 3. RanBP2 Depletion in RGG Cells by RNAi Causes Mitotic Arrest with Defective Spindle Assembly

RGG cells were transfected with siRNA oligonucleotides specific for RanBP2 or control oligonucleotides and analyzed after 86 hr unless otherwise indicated. The relatively long time required for depletion of RanBP2 may reflect a slow turnover rate for this protein.

(A) Total cell lysates were prepared from control (1) or RanBP2-depleted (2) cells. The lysates were subjected to SDS-PAGE and immunoblotted for RanBP2, RanGAP1, and Ran by using specific antibodies. Ran blot serves as a loading control. The arrow indicates SUMO-1 conjugated form of RanGAP1, while the asterisk represents unconjugated form of RanGAP1. We reproducibly observed that a substantial fraction of RanGAP1 became deconjugated from SUMO-1 in these samples, consistent with earlier reports indicating that RanBP2 protects SUMO-conjugated RanGAP1 from deconjugation by isopeptidases [28].

(B) Mitotic index was calculated at different intervals after control or RanBP2 siRNA transfection by counting immunopositive cells for MPM2 and phospho-H3 antibodies over total DAPI-positive cells. The numbers above bars indicate percentage of cells dead over total cells counted. In our hands, mitotic accumulation after RanBP2 depletion was more apparent in RGG cells than in other cell lines that we tested (e.g., HeLa, U2OS).

(C) Control and RanBP2-depleted cells were fixed with formaldehyde and analyzed. Pie charts show the percentage of mitotic cells with bi- and multipolar spindles. Lower panel shows immunofluorescence micrographs of bi- and multipolar spindles in RanBP2-depleted cells. The spindle poles are stained for Aurora A (red) by using antibodies and DNA with DAPI (blue).

(D) To examine kinetochore MT stability, control and RanBP2-depleted cells were subjected to cold treatment for 10 min before fixing with 4% paraformaldehyde. Cells were then stained for microtubules (red), centromeres (green), and DNA (blue) by using α-tubulin antibody, CREST antiserum, and DAPI, respectively.

ern blotting showed that RanBP2 levels were significantly decreased (>80% depletion compared to controls) 86 hr after transfection of siRNA oligonucleotides (Figure 3A). Although RanBP2-depleted cells still localized other nucleoporins to interphase NPCs (Figure S3A; see also [18]), RanGAP1 did not associate with the NE in the absence of RanBP2 (Figure S3B), consistent with earlier data indicating that RanBP2 binding is critical for its interphase targeting [19]. RanBP2-depleted RGG cells showed an increased mitotic index after 48 hr (Figure 3B), with over 15% of the cells accumulating in mitosis 96 hr after transfection. When chromosome distribution and spindle assembly were examined, RanBP2-depleted cells showed aberrant MT structures and an obvious failure of chromosome alignment on the metaphase plate (Figure S3, see also [18]). Simultaneous depletion of RanBP2 and Mad2 by RNAi reverted the elevation of mitotic index but also caused a dramatic increase in cells with micronuclei (data not shown), possibly reflecting inappropriate exit from mitosis without accurate chromosome segregation. These results indicate that RanBP2-depleted cells arrest in mitosis through activation of the mitotic spindle assembly checkpoint.

Although most spindles were multipolar after RanBP2 depletion, there was a striking similarity among the bipolar spindles formed in RanBP2-, Hec1-, and Nuf2-depleted RGG cells (Figures 1 and S1). Under all three conditions, spindles were longer than those of control cells with unaligned chromosomes: cells transfected with control oligonucleotides showed an average interpolar distance of 12.1 ± 1.2 μm (n = 25) prior to chromosome alignment, whereas RanBP2-depleted mitotic cells with bipolar spindles had an average interpolar distance of 16.6 ± 1.5 μm (n = 25), which was closer to Hec1-depleted cells (16.1 ± 2.0 μm) and Nuf2-depeleted cells (16.4 ± 1.8 μm). This similarity prompted us to examine the stability of kinetochore-MT attachment in RanBP2-depleted cells. As described previously for cells depleted of Nuf2 [6], we examined whether kinetochore MTs were sensitive to cold [20]. We subjected RanBP2-depleted cells to cold treatment for 10 min prior to fixation. The cells were stained with anti-α-tubulin antibodies and with CREST autoimmune sera that recognize centromeric proteins [21] (Figure 3D). While the control cells showed clear arrays of cold-stable kinetochore MTs, few kinetochore MTs were visible in the RanBP2-depleted cells. This observation suggests that a failure to form stable MT-kinetochore interactions may contribute to spindle defects in RanBP2-depleted cells. These findings are largely consistent with a recent report from Salinas et al. [18], who concluded that RanBP2 depletion causes kinetochore defects, resulting in a spindle checkpoint-dependent mitotic arrest. Since RanBP2 becomes mislocalized in mitotic cells after RNAi-mediated depletion of Hec1 and Nuf2 (Figure 2), our findings also suggest that some part of the failure to form stable MT-kinetochore interactions in the absence of Hec1 and Nuf2 [5, 6] may be related to an inability to correctly recruit the RanGAP1-RanBP2 complex under these circumstances.

We examined the localization of a number of kinetochore components after RanBP2 depletion. Kinetochores of RanBP2-depleted cells lacked Mad1, Mad2, CENP-E, and CENP-F, a kinetochore-associated passenger protein that interacts with CENP-E (Figure 2A, Figure S2). Mislocalization of these proteins was remarkable for two reasons: First, RanGAP1 and RanBP2 are not associated with kinetochores when Mad1 and Mad2 are present (Figure 2C), seemingly precluding direct binding of Mad1 and Mad2 to the kinetochores through the RanGAP1-RanBP2 complex. Our findings may thus imply an indirect requirement for the RanGAP1-RanBP2 complex in loading of Mad1 and Mad2 onto kinetochores. Changes in Ran-GTP levels may contribute to this phenotype, since recent experiments in *Xenopus* egg extracts demonstrated that Mad1 and Mad2 are released from kinetochores by elevated Ran-GTP concentrations [22]. Second, the accumulation of checkpoint proteins on kinetochores is typically closely coupled to activation of spindle checkpoint arrest pathways [12]. However, RanBP2-depleted cells showed a strong Mad2-dependent checkpoint arrest in mitosis without such accumulation (data not shown, see also [18]). Spindle checkpoint arrest of RanBP2-depleted cells in the absence of kinetochore accumulation of multiple checkpoint components is thus unusual, although not unprecedented [11]. Particularly, this phenotype is again reminiscent of the defects observed in Hec1-depleted cells, which fail to accumulate detectable levels of Mad1 and Mad2 at their kinetochores yet mitotically arrest in a Mad2-dependent fashion [11].

Our findings are largely consistent with those of Salinas et al. [18], who observed that CENP-F, dynein and checkpoint components (CENP-E, Mad1 and Mad2, and Zw10), fail to bind kinetochores in the absence of RanBP2. Through additional electron microscopy studies that showed altered kinetochore morphology, they concluded that RanBP2 depletion extensively or completely disrupts kinetochore formation. Surprisingly, our further analysis showed that three proteins associated with the kinetochore throughout mitosis (Hec1, Nuf2, and CENP-I [5, 7, 23]) retained their correct localization in RanBP2-depleted cells (Figure S2), arguing that many of the underlying kinetochore structures still assemble in the absence of RanBP2. Moreover, checkpoint components Bub1 and BubR1 also remained on kinetochores (not less than 85% of levels in control cells; Figure S2), arguing that many aspects of the cell cycle regulatory machinery remain intact in the absence of RanBP2.

RanBP2-depleted RGG cells revealed a high frequency of multipolar spindles among the mitotically arrested population (Figure 3C). We assessed the number of spindle poles by Aurora-A staining [24] in control and RanBP2 siRNA-treated cells to determine the proportion of cells that were multipolar: while 3% of mitotic RGG cells transfected with the control oligonucleotide formed multipolar spindles, 69% of the cells transfected with the oligonucleotide directed against RanBP2 were multipolar. These observations were interesting in light of previous reports that overexpression of the Ran-GTP binding protein RanBP1 leads to unscheduled centrosome splitting [25, 26].

To determine whether the additional poles contained centrosomes, RanBP2-depleted cells were stained with antibodies against human Centrin [27]. The number and distribution pattern of centrosomes both during interphase (data not shown) and mitosis (Figure 4) differed significantly compared to control RNAi-treated cells: more than 95 percent of the control mitotic cells possessed two centrosomes, each of which contained a pair of centrioles. These centrosomes were distributed to opposite spindle poles. The majority of multipolar cells in the RanBP2-depleted samples (72%) also possessed two pairs of centrioles. These centrioles were typically found within MT organizing centers (MTOCs) at spindle poles, although not all MTOCs had foci of Centrin staining. Spindles possessing single, unpaired centrioles within their MTOCs were also evident in RanBP2-depleted cells (17%). Some MTOCs within these cells lacked Centrin foci, indicating that they did not possess centrioles. Finally, around 11% of the multipolar RanBP2-depleted cells had more than two pairs of centrioles, not all of which were associated with obvious MTOCs. It is unclear whether the maldistribution of centrioles in RanBP2-depleted cells is a primary result of RanBP2 disruption or a secondary phenotype, caused indirectly by defects during earlier mitotic divisions in the absence of adequate levels of RanBP2.

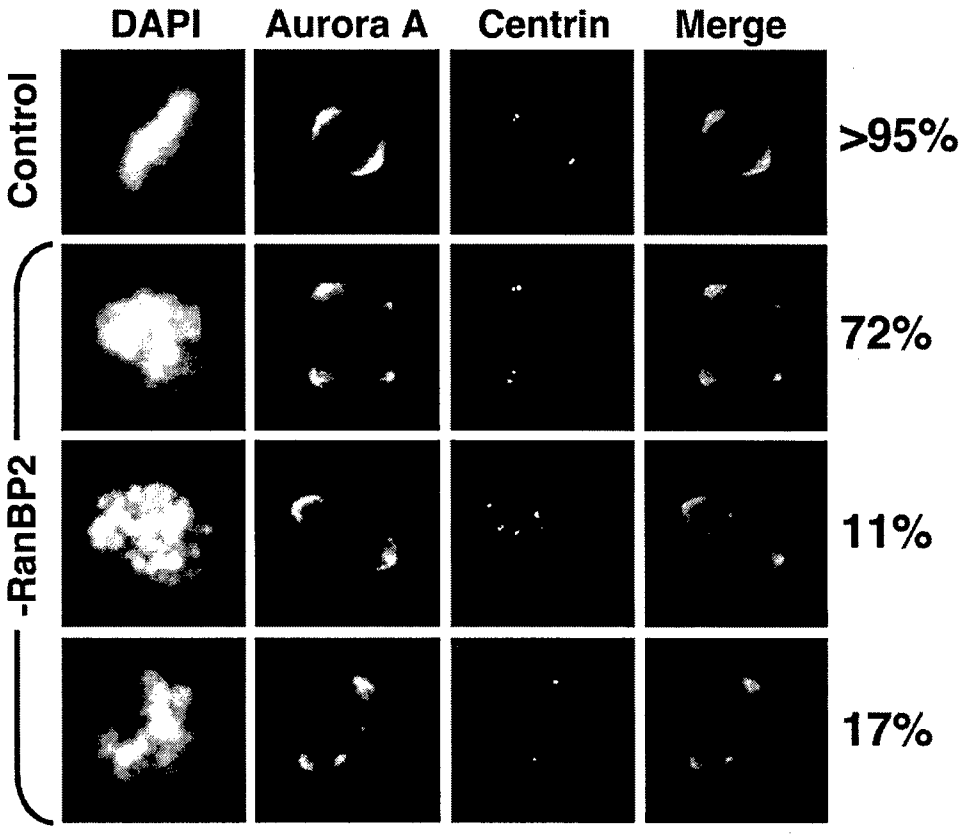

Figure 4. Disruption of Spindle Poles in RanBP2-Depleted Cells

RGG cells were transfected with siRNA oligonucleotides specific for RanBP2 or control oligonucleotides and processed for immunostaining after 86 hr. Cells were stained with Aurora A (green) and Centrin (red) antibodies for visualizing spindle poles and centrosomes, respectively. DNA was visualized by DAPI staining. Although over 95% of the control mitotic cells formed bipolar spindles where each pole contained a single pair of centrioles, the number of poles and centrioles in RanBP2-depleted cells varied widely. Numbers to the right of the RanBP2 depleted cells (−RanBP2) show the percentage of multipolar cells found in each of the major phenotypic classes (see text for further details).

However, the appearance of acentrosomal MTOCs in the majority of RanBP2-depleted cells clearly indicates that the RanGAP1-RanBP2 complex plays direct or indirect roles in spindle assembly beyond its function in kinetochore-MT attachment.

In summary, our results indicate that MT attachment is a critical event for recruitment of the RanGAP1-RanBP2 complex to kinetochores. The mitotic phenotypes of RanBP2- and Hec1- or Nuf2-depleted cells are very similar, possibly suggesting that RanBP2 recruitment may be an important aspect of Hec1 and Nuf2 function. The RanGAP1-RanBP2 complex plays an important role at the kinetochore by stabilizing MT attachments. It also regulates the recruitment of other components to the kinetochore; interestingly, these components include proteins such as Mad1 and Mad2, whose kinetochore accumulation patterns are different from those of the RanGAP1-RanBP2 complex. However, the RanGAP1-RanBP2 complex is not essential for Mad2-dependent checkpoint arrest. Spindle pole organization is disrupted in the absence of RanBP2, indicating a direct or indirect role of the RanGAP1-RanBP2 complex in additional aspects of spindle assembly and mitotic function.

Supplemental Data

Supplemental Data including Experimental Procedures and four figures are available at http://www.current-biology.com/cgi/content/full/14/7/611/DC1/

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2

The Role of Mitotic Checkpoint in Maintaining Genomic Stability

Song-Tao Liu[1], *Jan M. van Deursen*[2] *and Tim J. Yen*[1]
[1]Fox Chase Cancer Center, 7701 Burholme Avenue,
Philadelphia, PA 19111, USA

[2]Department of Pediatric and Adolescent Medicine,
Mayo Clinic, Rochester, MN 55905, USA

I. Introduction

"Considering that 2.5×10^8 cells are dividing in the human body at any given time, even if few errors occur, many genetically abnormal cells will be produced during the lifetime of an organism." Conly Rieder and Alexey Khodjakov wrote in a recent review (Rieder and Khodjakov, 2003). This is not an overstatement as faithful transmission of chromosomes is a tremendously challenging task during mitosis or meiosis. For somatic cells, defects in this process generate aneuploid cells that may turn into cancer. Aneuploid germ cells on the other hand can be the cause of infertility or birth defects such as Down Syndrome (Jallepalli and Lengauer, 2001; Nicklas, 1997).

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In a typical somatic cell cycle, M phase is usually the shortest when compared to S phase and the two gap phases, G1 and G2. Nevertheless, a mitotic cell undergoes a complex sequence of highly ordered morphological changes, which have amazed biologists for more than 100 years since Walther Flemming first described "mitosis" in 1882. In animal cells, the distinct changes of M phase starts with chromatin condensation in prophase. Duplicated centrosomes move away from each other and the interphase microtubule array disassembles to form a bipolar spindle. After nuclear envelope breakdown, chromosomes are spilled into cytoplasm where they are captured by spindle microtubules that probe the inner space of the cell by rapid cycles of growth and shrinkage. These dynamic microtubules become stabilized if they are attached to kinetochores, a macromolecular complex that is situated on opposite sides of the centromere of chromosomes. These attachments are essential for aligning the chromosomes at the spindle equator. Only when all the chromosomes in a cell are aligned (metaphase) will the signal to release the cell into anaphase be given.

Although it is clear that accurate chromosome segregation is dependent on highly complex mechanical processes that physically align and separate chromosomes (McIntosh *et al.*, 2002), these processes are also monitored by checkpoint regulatory systems (Pines and Rieder, 2001; Rieder and Salmon, 1998). The need for checkpoints is evident because attachment of chromosomes to the spindle is a stochastic process. Chromosomes do not synchronously attach to the spindle, but are independent events where individual chromosomes are captured by microtubules through chance encounters. This explains why not all chromosomes in a cell achieve alignment at the same time. This also underscores the need for a checkpoint system that ensures that cells do not prematurely exit mitosis until all of their chromosomes are aligned. The spindle assembly checkpoint is an evolutionarily conserved activity that monitors the kinetochore-microtubule interactions and prevents cells with even a single unattached kinetochore from exiting mitosis (Amon, 1999; Musacchio and Hardwick, 2002). Genetic and biochemical studies have revealed the target of spindle assembly checkpoint is Anaphase Promoting Complex/Cyclosome (APC/C), a multisubunit E3 ubiquitin ligase whose substrates such as securin and cyclin B must be degraded to allow disjunction of chromosomes and exit from mitosis (Harper *et al.*, 2002; Peters, 2002; Zachariae and Nasmyth, 1999).

The kinetochore is one of the most important structures established for cell division (Maney *et al.*, 2000; Rieder and Salmon, 1998). It not only provides the binding sites for spindle microtubules, but also harbors many microtubule motors and associated proteins to power and regulate the congression and separation of chromosomes. More interestingly, many

spindle assembly checkpoint proteins are found transiently localized at kinetochores, and kinetochores have been regarded as the major source of inhibitory checkpoint signals for the metaphase-anaphase transition.

In this chapter, we will discuss the most current information about the structure, composition, and assembly of kinetochores. We will focus on the role of checkpoint proteins in monitoring kinetochore:microtubule interactions and models of the signal transduction pathways that link unattached kinetochores to the APC/C. The following part of this review will deal with the roles of checkpoint proteins in normal development and in tumorigenesis. Finally, we will briefly talk about the important questions still awaiting for answer. We want to stress that although we will mainly talk about research on mitosis, basic principles also apply to the regulation of meiosis.

II. The Kinetochore is a Complex Structure for Cell Division

A. The Structure and Function of Kinetochores

The kinetochore is a macromolecular complex that is assembled from centromeres. Readers are referred to several recent excellent reviews that discuss centromere structure (Cleveland *et al.*, 2003; Mellone and Allshire, 2003). Electron microscopic images of kinetochores from animal cells reveal disk-shaped structure with four morphologically distinct domains (Rieder, 1982; Rieder and Salmon, 1998). Juxtaposed to the centromeric heterochromatin is an electron dense inner plate, sometimes hard to distinguish from chromatin. An electron-dense outer plate of about 35–40 nm thickness is separated from inner plate by an electron-lucent middle layer (or central zone, interzone) of 15–35 nm thickness. However, under a different fixation condition that employed high-pressure freezing and freeze substitution, this interzone was not discernible (McEwen *et al.*, 1998). Emanating from the surface of the outer plate is the "fibrous corona" which is a loose meshwork of fibrillar projections. It is likely that this structure contributes to the functional organization of the kinetochore that includes microtubule attachment, force generation, and a checkpoint system that monitors these activities.

B. CENP-E: a Kinetochore Associated Kinesin-like Protein

CENP-E (Centromere Protein E) was the first kinesin-like protein that localized specifically to kinetochores in mitotic cells (Yen *et al.*, 1991, 1992).

Immuno-EM studies localized CENP-E at the fibrous corona on the surface of the outer kinetochore plate (Cooke *et al.*, 1997). CENP-E is attached to the kinetochore through a domain at its carboxyl terminus (Chan *et al.*, 1998). This configuration orients its amino-terminal motor-like domain away from the chromosome in order to maximize its interactions with microtubules. CENP-E contains a second microtubule binding domain at its extreme carboxyl-terminus but its importance to function remains to be examined. Functional analysis of human CENP-E by antibody injection, dominant negative mutants and anti-sense oligonucleotides revealed that it is only essential for monopolar chromosomes that normally exist transiently to convert to bipolar attachments (Chan *et al.*, 1998; McEwen *et al.*, 2001; Schaar *et al.*, 1997; Yao *et al.*, 2000). High resolution time-lapse studies showed that CENP-E was essential for chromosomes that are positioned near a pole at the onset of mitosis to establish bipolar attachments (McEwen *et al.*, 2001). In the absence of CENP-E, these chromosomes maintain a monopolar attachment because their sister kinetochores are unable to capture the rare microtubule that originates from the opposite pole. For chromosomes that are positioned near the center of the spindle, the high frequency of encounters with microtubule can compensate for the loss of CENP-E as other (yet to be determined) kinetochore components are able to establish bipolar attachments. Quantitative EM analysis show that kinetochores lacking CENP-E are able to establish near normal ($\sim 73\%$) the number of microtubule connections. Nevertheless, these connections are defective as tension between the sister kinetochore is never developed. This observation suggests that CENP-E is responsible for generating kinetochore tension and must therefore contribute towards poleward force generation.

The caveat to these functional studies is that the methods used to inhibit CENP-E expression or function may not reflect the null state. Recent studies of cells derived from CENP-E null mouse embryos showed that they exhibited similar chromosome defects as reported for Hela cells (Putkey *et al.*, 2002). Likewise, *in vitro* studies using *Xenopus* egg extracts depleted of CENP-E also showed an accumulation of monopolar chromosomes (Abrieu *et al.*, 2000). The combined data suggests that CENP-E becomes critically important in areas of low microtubule density where kinetochores must efficiently capture microtubules to achieve biorientation. On the other hand, CENP-E is dispensable for attachment if kinetochores encounter microtubules at high frequencies. There must be other kinetochore components that are responsible for these connections but their identities remain to be determined.

In addition to CENP-E, kinetochores also contain other molecular motors such as dynein and MCAK (a kinesin-related protein that does not behave as a motor but is a microtubule destabilizing enzyme)

(Pfarr *et al.*, 1990; Steuer *et al.*, 1990; Wordeman and Mitchison, 1995). Kinetochores also contain microtubule binding proteins CLIP170 and Orbit/Mast (Dujardin *et al.*, 1998; Maiato *et al.*, 2002). It is unclear if any of these microtubule interacting proteins are responsible for the microtubule attachments that are seen when CENP-E is depleted from kinetochores. This question will be addressed by depleting different combinations of these proteins.

III. The Mitotic Checkpoint

A. Overview

The spindle assembly checkpoint can be viewed as a signal transduction cascade whereby a localized signal generated from an unattached kinetochore is amplified to inhibit the cellular targets that are required for initiating the transition from metaphase to anaphase (Fig. 1). Genetic and biochemical analysis have shown that the target of the checkpoint is the Anaphase Promoting Complex/Cyclosome (APC/C), a multisubunit E3 ubiquitin ligase that specifies the degradation of specific proteins in order to drive cells out of mitosis (Harper *et al.*, 2002; Peters, 2002). The models for how APC is inhibited by checkpoint proteins will be discussed below.

The molecular components of the mitotic checkpoint are specified by seven evolutionarily conserved genes that were first identified in budding yeast (Hoyt *et al.*, 1991; Li and Murray, 1991; Weiss and Winey, 1996). Homologs of BUB1, BUB3, MAD1, MAD2, MAD3, and MPS1 have been shown to be essential for establishing the checkpoint response in all eukaryotes examined to date (Abrieu *et al.*, 2001; Basu *et al.*, 1999; Cahill *et al.*, 1998; Chan *et al.*, 1999; Chen *et al.*, 1996; Kitagawa and Rose, 1999; Li and Benezra, 1996; Liu *et al.*, 2003a; Luo *et al.*, 2002; Taylor and McKeon, 1997). In addition, the mammalian ortholog of the yeast nuclear export factor, Rae1, has been shown to be also important for the spindle checkpoint (Babu *et al.*, 2003). Recent studies also documented that ZW10 and ROD, two proteins that have no counterparts in *S. cerevisiae* but are conservative amongst metazoans, are also essential for the checkpoint (Chan *et al.*, 2000; Scaerou *et al.*, 2001). The appearance of ZW10 and ROD in metazoans may reflect the need for additional checkpoint proteins to monitor increasingly complex activities that are associated with the kinetochore.

Where along the checkpoint pathway these proteins act remain an active area of investigation. The finding that all of these proteins preferentially bind to unattached kinetochores suggested that they participate in monitoring kinetochore microtubule interactions (Chan *et al.*, 1999, 2000; Chen *et al.*, 1996; Jablonski *et al.*, 1998). However, some of these

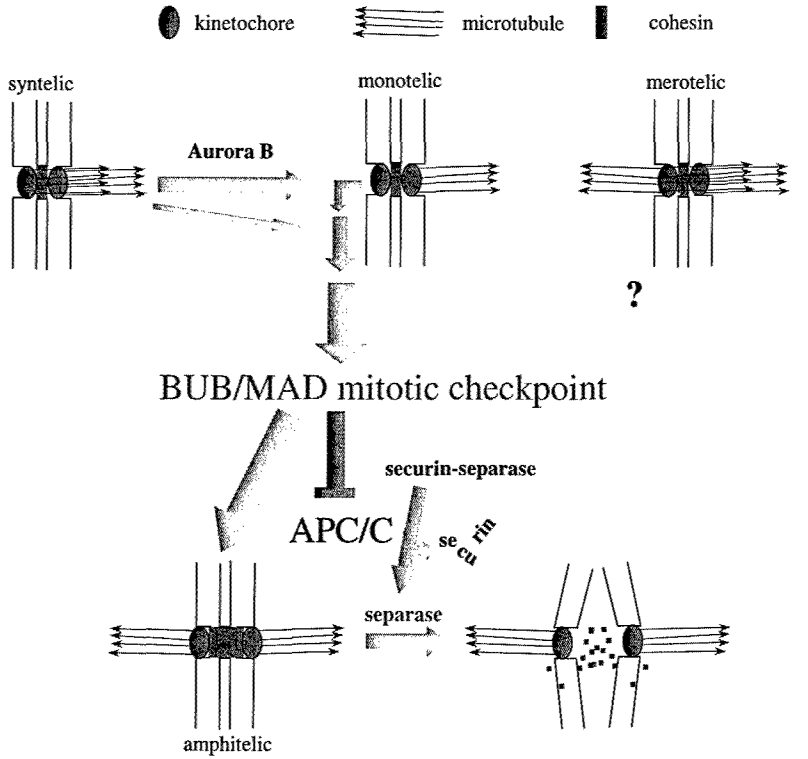

Figure 1 Mitotic checkpoint as a signal transduction pathway. Unattached kinetochores in monotelic chromosomes initiate "wait anaphase" signal transduction and activate mitotic checkpoint, leading to the inhibition of APC/C by BUB/MAD checkpoint proteins. The status monitored at kinetochores may be microtubule occupancy or tension. Syntelic chromosomes may be transformed into mototelic by Aurora B, or the lack of tension on their kinetochores may directly start the checkpoint. It is suggested merotelic attachment may not be able to activate the mitotic checkpoint. Only when all the chromosomes in a mitotic cell reach amphitelic attachment and tension develops between sister kinetochores will the mitotic checkpoint stop and APC/C catalyze the ubiquitination and degradation of securin. The released separase then cut the cohesion between sister chromatids, thus finishing the metaphase-anaphase transition (see color plate).

proteins may also be directly involved in inhibiting the APC (Fang, 2002; Sudakin *et al.*, 2001; Tang *et al.*, 2001). Thus, checkpoint proteins such as Mad2 and BUBR1 may act both at kinetochores and downstream of kinetochores.

The ability of unattached kinetochores to inhibit mitotic exit has been long recognized. Indeed early observations suggested that unattached kinetochores may send negative signals to prevent premature anaphase (McIntosh, 1991; Zirkle, 1970). This idea received direct experimental support through a series of experiments by Conly Rieder and Bruce Nicklas'

labs (Li and Nicklas, 1995, 1997; Nicklas *et al.*, 1995; Rieder *et al.*, 1994; Rieder *et al.*, 1995). Rieder *et al.* documented that anaphase onset always occurred about 23 min after the last kinetochore became attached to microtubules and aligned at the metaphase plate (Rieder *et al.*, 1994). They also observed that it may take up to 3 h for the last monooriented chromosome to achieve bipolar attachment. However, if the unattached kinetochore of the monooriented chromosomes is ablated with a pulse of laser, the cell entered anaphase about 20 min later, the same interval that was seen in a normal cell (Rieder *et al.*, 1995). Similar results were obtained by Nicklas's lab who showed that repeated detachment of a chromosome in grasshopper spermatocytes delayed anaphase onset indefinitely (Nicklas *et al.*, 1995). Furthermore, if an external force is applied to a monooriented chromosome so that the sister kinetochores are under tension as in the case for a bioriented chromosome, the cell entered anaphase (Li and Nicklas, 1995). Collectively, these experiments show that unattached kinetochores emit an inhibitor of anaphase rather than the ability of aligned chromosomes to emit a positive factor to initiate anaphase.

B. Tension and Microtubule Occupancy

Although it is accepted that unattached kinetochores emit the inhibitory signal that delays anaphase onset, what is the nature of the defect that activates the checkpoint? Kinetochores of properly aligned chromosomes are saturated with microtubules and tension develops between the sister kinetochores as the opposing poleward forces try to pull them apart. Results from the micromanipulation experiments conducted by Nicklas' lab suggested that the checkpoint was sensitive to the level of tension between sister kinetochores. However, the laser ablation experiments indicated that the checkpoint might be monitoring microtubule occupancy as there is little tension between the kinetochores of a monooriented chromosome but cells are able to exit mitosis when the unattached kinetochore is destroyed (Rieder *et al.*, 1995). These data suggest that both tension and microtubule occupancy can regulate the spindle assembly checkpoint, but preference for one over the other may depend on mitosis versus meiosis, cell types and organisms (Zhou *et al.*, 2002b).

The notion that the checkpoint is sensitive to only tension or microtubule occupancy may be inaccurate as both criteria must be fulfilled before a cell can exit mitosis. Cells exposed to microtubule inhibitors (taxol, noscapine and low dose of vinblastine) or low temperature will arrest in mitosis despite the fact that their chromosomes appear to be "aligned" at the spindle equator (Shannon *et al.*, 2002; Skoufias *et al.*, 2001; Waters *et al.*, 1998; Zhou *et al.*, 2002a). As these treatments suppress microtubule dynamics

without blocking polymer formation, kinetochores are able to achieve near maximum number of microtubule attachments. Despite this, kinetochores cannot generate tension as the suppression of microtubule dynamics prevents poleward forces from being generated. Mad2 checkpoint protein which has been shown to preferentially bind to unattached kinetochores is no longer detected at kinetochores in taxol treated cells. This suggests that Mad2 is sensitive to microtubule occupancy at kinetochores but not to tension. Although in normal cells, the release of Mad2 from fully attached kinetochores signifies the onset of anaphase, taxol treated cells remain arrested in mitosis because their kinetochores lack tension. What are the checkpoint proteins that are responsible for monitoring tension remains unresolved, but Bub1 and BubR1 have been postulated to play this role as they are present at tensionless kinetochores (Skoufias *et al.*, 2001; McEwen *et al.*, 2001). It should be pointed out that unlike Mad2, neither Bub1 or BubR1 completely dissociate from fully attached kinetochores in normal metaphase cells that are about to enter anaphase (Jablonski *et al.*, 1998). The mere presence of Bub1 and BubR1 is therefore not a reliable indicator of the checkpoint status of cells. If these proteins are involved in monitoring tension, it is likely that it is achieved through regulation of their kinase activities rather than localization.

However, a recent study found evidence that the loss of kinetochore tension is insufficient to block anaphase onset. When tension at kinetochores in grasshopper spermatocytes was removed by micromanipulation, the number of kinetochore microtubules decreased by 60% (King and Nicklas, 2000). This is consistent with earlier studies showing that tension altered the stability of kinetochore microtubules (Nicklas and Ward, 1994; Zhai *et al.*, 1995). Mad2 levels were reduced to approximately 17% of that found at a fully unattached kinetochore. Despite the lack of tension and reduced microtubule occupancy, the spermatocytes were only able to delay anaphase onset rather than arrest. These cells are believed to be unable to arrest because their kinetochores, perhaps due to a reduced amount of Mad2, are unable to generate sufficient amounts of "wait anaphase" signal to sustain a prolonged arrest. These authors postulated that the microtubule attachment determines the strength of the output of the checkpoint signal, but tension may be essential to saturate microtubule binding at kinetochores and completely turn off the checkpoint (Nicklas *et al.*, 2001).

The interplay between microtubule attachment and tension is complex and it may be difficult to dissect their individual contributions to the checkpoint. However, it now appears that the Ipl1/Aurora B kinase may link tension to microtubule attachments. Yeast mutants with unreplicated chromosomes (*cdc6*) or chromosomes with defective cohesion (*mcd1*) arrest in mitosis because their kinetochores lack tension even though they are

attached to microtubules (Biggins and Murray, 2001). This arrest is dependent on the Ipl1 kinase even though Ipl1 is not required for cells to arrest in mitosis in the presence of microtubule inhibitors that prevent kinetochore attachments. While one interpretation is that Ipl1 is a checkpoint protein that monitors tension, another view is that Ipl1p facilitates biorientation by promoting turnover of the microtubule connections at kinetochores that lack tension (Tanaka, 2002; Tanaka *et al.*, 2002). Ipl1 may be part of a self-correcting mechanism that prevents syntelic attachments, a situation whereby sister kinetochores become attached to the same pole. As syntelic attachments should fulfill the microtubule occupancy checkpoint, it is critical that this defect be corrected in order to prevent nondisjunction. This can be accomplished if the absence of tension activates Ipl1 kinase to release microtubule attachments. In this scenario, Ipl1p indirectly activates the checkpoint in the absence of kinetochore tension by catalyzing the release of microtubules. This idea can also account for why Ipl1p is not required for the checkpoint arrest induced by the loss of microtubule attachments.

The yeast data have now been confirmed by studies in mammalian cells that show inhibition of Aurora B kinase prevents cells from arresting in mitosis in the presence of taxol (no tension) but not nocodazole (no microtubule occupancy) (Ditchfield *et al.*, 2003; Hauf *et al.*, 2003). The mechanism by which Aurora B kinase monitors tension is not known, but its localization between sister kinetochores suggests that it may be sensitive to centromere stretching. The link between Aurora B and the spindle checkpoint is not entirely clear as there is a discrepancy as to whether inhibition of Aurora B kinase interferes with the ability of Mad2 and BubR1 to assemble onto kinetochores (Ditchfield *et al.*, 2003; Hauf *et al.*, 2003). Regardless of this discrepancy, the reason for why these cells are able to arrest in nocodazole but not taxol remains to be sorted out. One explanation hinges on the assumption that unattached kinetochores lacking Aurora B are unable to generate sufficient levels of "wait anaphase" signal. However, the collective output from all of the unattached kinetochores in nocodazole treated cells may be sufficient to arrest cells in mitosis. In contrast, in the absence of Aurora B, the output from kinetochores with attachments but no tension might be even lower than a fully unattached kinetochore. Consequently, the collective amount of inhibitory signal generated from kinetochores of taxol treated cells may be insufficient to arrest mitosis.

C. CENP-E and the Mitotic Checkpoint

Functional studies of CENP-E revealed that its activity was also monitored by the spindle assembly checkpoint as Hela cells lacking CENP-E functions

were arrested in mitosis (McEwen *et al.*, 2001; Schaar *et al.*, 1997; Yao *et al.*, 2000). A molecular connection between the checkpoint and CENP-E was revealed when it was discovered that CENP-E interacts with hBUBR1, a protein kinase that exhibited similarities with the MAD3 and BUB1 spindle checkpoint proteins in budding yeast (Chan *et al.*, 1998, 1999). Indeed, disruption of hBUBR1 function in Hela cells abrogated their ability to arrest in mitosis in response to microtubule destabilizing drugs (Chan *et al.*, 1999). More importantly, it was shown that the mitotic arrest induced by the disruption of CENP-E function depended on hBUBR1. Given that hBUBR1 was localized to kinetochores, the combined results supported a model where the kinetochore activities of CENP-E was monitored by hBUBR1 kinase. We envisioned hBUBR1 to behave as a mechanosensor where its checkpoint activity was regulated by the interactions between CENP-E and microtubules. Depending on these interactions, hBUBR1 kinase activity may be allosterically regulated to either initiate or silence the checkpoint signal from kinetochores (Chan and Yen, 2003).

The ability of cells to arrest in mitosis in response to inhibition of CENP-E functions is not universal. Xenopus egg extracts depleted of CENP-E fail to align their chromosomes, yet they are unable to maintain a mitotic arrest when compared to extracts treated with microtubule inhibitors (Abrieu *et al.*, 2000). This observation supports the idea that CENP-E is an integral component of the checkpoint. However, the reason for why kinetochores lacking CENP-E fail to establish a checkpoint arrest is likely due to the absence of the Mad2 checkpoint protein at these kinetochores. This contrasts with studies in Hela cells where unattached kinetochores that were depleted of CENP-E retained Mad1, Mad2 (McEwen *et al.*, 2001). Thus, the disparity between how egg extracts and Hela cells respond to the loss of CENP-E may be attributed to how CENP-E affects the assembly of checkpoint proteins at kinetochores.

The reason for the difference between egg extracts and Hela cells is unresolved, but a likely possibility is that it is due to fundamental differences in how kinetochores are assembled between embryonic and somatic cells. Unfortunately, this explanation is clouded by the recent report where hepatic cells obtained from a conditional CENP-E knockout mouse also failed to arrest in mitosis in the presence of unaligned chromosomes (Putkey *et al.*, 2002). However, it is unknown whether the length of the mitotic delay exhibited by these cells in response to loss of CENP-E is the same as their responses to nocodazole or taxol. The apparent discrepancy between how Hela cells and the mouse hepatocytes respond to the inactivation of CENP-E may lie in inherent differences in the duration of the checkpoint arrest.

D. Monitoring Microtubule Occupancy

The ability of Mad2 to preferentially bind to kinetochores that lack microtubule attachments suggests that one of its checkpoint functions is to monitor microtubule attachments. Indeed Mad2 is essential for arresting cells in mitosis when their kinetochores lack microtubule attachments. However, two recent studies in Hela cells suggest that there may be redundant mechanisms that monitor microtubule attachments (Liu *et al.*, 2003b; Martin-Lluesma *et al.*, 2002). Hela cells were found to delay in mitosis for a considerable amount of time despite the lack of detectable Mad2 at their kinetochores. In previous studies, direct inhibition of Mad2 caused cells to accelerate out of mitosis within 10 min of its inhibition (Gorbsky *et al.*, 1998). Given that Mad2 appears to act both at kinetochores where it contributes towards initiating the signaling cascade and downstream from kinetochores by directly inhibiting the APC, it was impossible to discern these two activities by directly inhibiting Mad2. What makes these two recent studies intriguing is that they selectively disrupted Mad2 functions at kinetochores by preventing its ability to assemble there. When HEC1 was prevented from assembling onto kinetochores, Mad1, Mad2, and hMPS1 also failed to bind to kinetochores (Martin-Lluesma *et al.*, 2002). Similarly, Mad1 and Mad2 (the localization of hMPS1 was not tested) were unable to bind to kinetochores when CENP-I was prevented from assembling there (Liu *et al.*, 2003b). Interestingly, injection of Mad2 antibodies into the mitotically delayed cells resulted in their rapid exit from mitosis. The caveat of these studies is whether the arrest is mediated by residual Mad2 that is below the limits of detection. With this in mind, these latest findings suggest that the kinetochore localization of Mad1, Mad2 and hMPS1 are not essential for cells to arrest in mitosis in response to loss of microtubule attachment. The presence of hBUBR1, hBUB1 and hZW10 at these kinetochores suggests that they are likely responsible for maintaining the arrest. Regardless, these findings increase the complexity by which checkpoint proteins monitor kinetochore attachments as it suggests the presence of redundant monitoring systems. As described for Aurora B, it is possible that no individual kinetochore lacking Mad1, Mad2, and hMPS1 is able by itelf to generate sufficient amounts of "wait anaphase" signal to arrest cells in mitosis. However, the collective output from many unattached kinetochores are required to achieve the threshold level to arrest mitosis.

E. Checkpoint Inhibition of the APC/C

The target of the mitotic checkpoint is the APC/C. Two models have been proposed to explain how checkpoint proteins inhibit the APC/C.

1. Anaphase Promoting Complex/Cyclosome (APC/C)

APC/C is an evolutionarily conserved multisubunit E3 ubiquitin ligase that was originally identified in clam oocytes, frog egg extracts, and yeast (Irniger *et al.*, 1995; King *et al.*, 1995; Sudakin *et al.*, 1995). It is composed of at least eleven subunits (Peters, 2002), with new subunits being discovered (Hall *et al.*, 2003). Many proteins have been found ubiquitinated by APC/C and targeted to 26S proteasome for degradation (Harper *et al.*, 2002; Peters, 2002). However the two key substrates whose degradation is critical for sister chromatid separation and mitotic exit are securin/pds1 and cyclin B, respectively. Securin binds to and inhibits separase/ESP1, a cysteine protease that is believed to degrade the Scc1/Mcd1 subunit of the cohesin complex in order to dissolve sister chromatid cohesion (Nasmyth *et al.*, 2000). Cyclin B associates and activates Cdc2 kinase whose activity is essential for maintaining cells in mitosis. The APC/C relies on specificity factors such as CDC20 and CDH1 to recruit substrates to the APC/C. APC/C^{CDC20} activity is required for the metaphase to anaphase transition as it ubiquitinates proteins such as securin and mitotic cyclins. On the other hand, APC/C^{CDH1} promotes progression through the late stages of mitosis by ubiquitinating cyclin B and CDC20. Readers are referred to several comprehensive reviews for more information on APC/C (Harper *et al.*, 2002; Peters, 2002; Zachariae and Nasmyth, 1999).

2. Sequestration Model

The molecular link between the mitotic checkpoint and the APC/C was established when genetic and biochemical evidence from yeast and frog egg extracts, respectively, showed that Mad2 can bind CDC20 and thus prevent its ability to recruit substrates to the APC/C (Fang *et al.*, 1998; Hwang *et al.*, 1998). This finding coupled with the *in vivo* observation that Mad2 undergoes rapid rates of exchange at kinetochores (Howell *et al.*, 2000) led to a molecular model for how unattached kinetochores inhibit the APC/C (Shah and Cleveland, 2000; Yu, 2002). This model suggests that Mad2 undergoes a conformational change through a transient interaction with kinetochores. Upon release from kinetochores, the "activated" Mad2 sequesters CDC20 and thus prevents activation of the APC/C. A critical feature of this model is that all of the steps along the checkpoint pathway (initiation, amplification and target inhibition) are intimately linked through the pool of Mad2 that cycles through the kinetochore. In a recent study, PtK1 cells were injected with a Mad1 mutant that is unable to bind kinetochores but retains its ability to bind to Mad2 (Canman *et al.*, 2002). The injected cells were found to exit mitosis despite the presence of Mad2 at their kinetochores. Furthermore, the kinetochore associated

Mad2 exhibited the same dynamic properties as in control cells. One interpretation is that the Mad1 mutant is sequestering the Mad2 that is released from kinetochores so that it cannot bind to CDC20. However, based on the results from the HEC1 and CENP-I studies where Mad2 acts at two discrete steps along the checkpoint pathway, an alternative view is that mitotic exit is induced because the Mad1 mutant acts on the pool of Mad2 that is directly responsible for inhibiting the APC/C. The assumption here is that the Mad2 that is released from kinetochores is unimportant for inhibiting the APC/C. Indeed, there is currently no data that document the fate of the Mad2 molecules that are released from kinetochores.

A similar mechanism for how hBUBR1 inhibits the APC has been postulated. *In vitro* binding assays and yeast two hybrid data showed that hBUBR1 can bind to CDC20 (Fang, 2002; Tang *et al.*, 2001; Wu *et al.*, 2000). This observation has fueled speculation that hBUBR1 may act in parallel with Mad2 to sequester CDC20 and prevent activation of the APC/C. Using an assay whereby APC/C activity is dependent upon exogenous CDC20, addition of hBUBR1 or Mad2 prevented activation of APC/C. The caveat of this experiment is that the APC/C used in these studies are from interphase cells and thus not the physiologically relevant substrate for inhibition. Indeed, when these assays were performed with APC/C purified from mitotic cells, recombinant hBUBR1 failed to inhibit its activity (Tang *et al.*, 2001).

3. APC/C Sensitization Model

An alternative to the sequestration model proposes that the checkpoint directly inhibits the APC/C. This idea originated from the discovery in Hela cells of a factor that selectively inhibited mitotically active APC/C (Sudakin *et al.*, 2001). Lysates prepared from mitotically arrested Hela cells were fractionated to identify factors that inhibited APC/C activity. This led to the identification of the Mitotic Checkpoint Complex MCC, which consists of the checkpoint proteins hBUBR1, hBUB3, CDC20 and Mad2. The evidence that the MCC is the physiologically relevant inhibitor of the APC/C are based on the following observations:

1. APC/C that is purified from mitotically arrested cells is either free or associated with the MCC. The free APC/C exhibits ubiquitin ligase activity while the pool that is associated with the MCC is inactive.
2. All studies that showed Mad2 can inhibit the APC/C relied on the use of recombinant Mad2 protein at levels that were at least an order of magnitude higher than endogenous levels.

3. Direct comparison of the APC/C inhibitory activity between purified MCC and recombinant Mad2 showed that the MCC was >3000-fold more potent of an inhibitor than recombinant Mad2.
4. Based on the titration experiments, the intracellular concentration of "free" pool of Mad2 would have to be >3000-fold higher than MCC in order for it to be an effective inhibitor. Although there is a large pool of Mad2 that is not associated with the MCC in Hela cells, this pool is no more than 25 to 50-fold higher concentration than the amount of Mad2 that is associated with MCC. Furthermore, fractions containing this pool of Mad2 exhibits no detectable APC/C inhibitory activity.
5. The concentration of MCC is in near equal stoichiometry with the APC/C.

The discovery of MCC also challenged the prevailing view that the inhibitor of the APC/C is directly generated from unattached kinetochores. The MCC was isolated in mitotic Hela cells, but it was found to be present and fully active in interphase cells. As kinetochores are not even assembled during interphase, MCC formation must occur independently of kinetochores. Although MCC synthesis and activity were not subject to cell cycle regulation, it preferentially inhibits mitotic APC/C. The need for a preformed pool of inhibitor is apparent because APC/C is phosphorylated and rapidly activated at the onset of mitosis. By necessity, the inhibition of the APC/C by the MCC must also be reversible so that cells can exit mitosis. It is believed that the interaction between MCC and the APC/C is labile unless the presence of unattached kinetochores stabilizes the interaction. This is indirectly supported by reconstitution experiments that showed APC/C activity in lysates prepared from mitotically arrested Hela cells cannot remain suppressed as ubiquiting ligase activity is reproducibly reactivated after an initial lag (Sudakin *et al.*, 2001). This lag, which represents the checkpoint inhibited APC/C activity, can be extended when chromosomes (unattached kinetochores) are added to these extracts. As neither MCC inhibitory activity or CDC20 stimulatory activity are stimulated by chromosomes, the likely target of kinetochores appears to be the APC/C.

The combined data suggests a model whereby the "wait anaphase" signal that is generated by kinetochores does not directly inhibit the APC/C but rather sensitizes the APC/C to prolonged inhibition by the MCC. This model predicts that the checkpoint pathway can be separated into discrete steps that are acted on by different components of the checkpoint. The checkpoint proteins that are localized at kinetochores initiate a signal in response to improper microtubule attachments. This signal must be amplified, perhaps through a kinase cascade, to target

a large population of APC/C for prolonged inhibition by the cytosolic pool of MCC.

Presently, it is unclear why kinetochores and the MCC share many of the same proteins especially when the biochemical activities required to generate the "wait anaphase" signal and to bind and inhibit the APC/C are likely to be quite different. One possibility is that hBUBR1, hBUB3, CDC20, and Mad2 do not form the MCC when they are associated at kinetochores. For example, hBUBR1 is believed to interact with CENP-E when it is associated at kinetochores even though it is part of the MCC in the cytoplasm. By altering protein interactions, it may be possible for a single protein to adopt multiple functions.

The existence of the MCC in Hela cells has now been independently confirmed by others. MCC-like complexes have also now been identified in fission, budding yeasts and frog egg extracts (Chen, 2002; Fraschini *et al.*, 2001; Millband and Hardwick, 2002). In budding yeast, the formation of the MCC was also found to be independent of kinetochores (Fraschini *et al.*, 2001). Whether these complexes represent physiological inhibitors of the APC/C in those species remains to be tested.

The existence of an inhibitor of the APC/C whose activity is not dependent on kinetochores is consistent with the observations that Mad2 appears to act at two distinct steps along the checkpoint pathway. When Mad2 is depleted from kinetochores by disruption of HEC1 or CENP-I, cells are able to delay mitosis in a Mad2 dependent manner (Liu *et al.*, 2003b; Martin-Lluesma *et al.*, 2002). The requirement for Mad2 in these cases likely reflects the role of the MCC in inhibiting the APC/C. If kinetochores lacking Mad2 are only able to generate a low level of "wait anaphase" signal, it is unable to sensitize the APC/C to prolonged inhibition by the MCC.

IV. Mutations in Mitotic Checkpoint Proteins and Tumorigenesis

A. Mad2, Bub3, and Rae1 are Haplo-Insufficient for Tumor Suppression

An estimated 85% of human cancer cells possess an abnormal number of chromosomes. Thus, researchers have long been curious about the role of aneuploidy in the multi-step cancer process. Indeed, whether chromosomal instability and aneuploidy is the cause or merely a consequence of cancer remains a central question in cancer biology. Since the discovery of the spindle assembly checkpoint in yeast, researchers have speculated that loss of this checkpoint in humans would play a key role in the development of

aneuploidy in human cancers. Several laboratories have tested this hypothesis by disrupting key components of the spindle assembly checkpoint in the mouse by gene targeting and embryonic stem cell technology. To date, Mad2, Bub3, and Rael knockout studies have been reported each showing that complete mitotic checkpoint protein loss results in early embryonic death (Babu *et al.*, 2003; Dobles *et al.*, 2000; Kalitsis *et al.*, 2000). In all cases, null embryos are indistinguishable from wildtype embryos until the blastocyst stage (32-cell stage), but subsequently fail to expand their inner cell mass of pluripotent mitotic cells and begin to degenerate.

At first, the embryonic lethality seemed to limit the usefulness of gene knockout models for studying the connection between chromosomal instability and cancer, but recent studies of heterozygous knockout mice have proven otherwise. Although Mad2, Bub3, and Rael heterozygous knockout mice have no overt phenotype, cells from these mice show markedly impaired mitotic checkpoint activation and mis-segregate chromosomes at higher than normal rates (Babu *et al.*, 2003; Michel *et al.*, 2001). Consistent with a functional role for chromosomal instability in cancer development, Mad2, Bub3, and Rael heterozygous knockout mice are more susceptible to formation of spontaneous and/or carcinogen-induced lung tumors than normal mice. These observations suggest that mitotic checkpoint genes function as haplo-insufficient tumor suppressors. This class of tumor-suppressor genes may be frequent targets during cell transformation processes because inactivation of only one allele or a reduction in gene expression level is sufficient to advance the multi-step process of cancer (Fero *et al.*, 1998). One mechanism that should be very effective in reducing mitotic checkpoint gene expression levels involves hypermethylation of CpG islands, an epigenetic means of DNA modification that is common in human cancers (Laird, 2003). Indeed, a recent study from Shichiri and coworkers shows that epigenetic silencing of the mitotic checkpoint genes Bub1 and BubR1 is a frequent event in aneuploid human colon carcinomas, with 30% of the carcinomas exhibiting at least a two-fold reduction in Bub1 or BubR1 expression (Shichiri *et al.*, 2002). Such Bub1 or BubR1 reductions are expected to predispose cells to chromosomal mis-segregation in mitosis and may have established the aneuploidy in the tumors. Another mechanism by which reduced expression of mitotic checkpoint genes could be accomplished involves the loss of whole chromosomes in mitosis. For instance, as a result of chromosomal mis-segregation, a cell may lose a chromosome containing a mitotic checkpoint gene. Such a mis-segregation event might be caused by genotoxic agents (Hesterberg and Barrett, 1985; Hunt *et al.*, 2003), mitotic checkpoint gene mutations, or might simply be a fortuitous event. Knowing that the mammalian mitotic checkpoint system is extremely sensitive to under-expression of its components, it is not surprising that many studies have

concluded that inactivating point mutations in mitotic checkpoint genes are rare events in human tumors with chromosomal instability (Cahill *et al.*, 1998, 1999; Gemma *et al.*, 2001; Hempen *et al.*, 2003; Hernando *et al.*, 2001; Imai *et al.*, 1999; Lin *et al.*, 2002; Mimori *et al.*, 2001; Nakagawa *et al.*, 2002; Nomoto *et al.*, 1999; Olesen *et al.*, 2001; Ouyang *et al.*, 2002; Percy *et al.*, 2000; Reis *et al.*, 2001; Sato *et al.*, 2000; Tsukasaki *et al.*, 2001). More recent studies have identified several novel components of the mitotic checkpoint that were not included in the reported screenings of human tumors for mitotic checkpoint mutations (Babu *et al.*, 2003; Cahill *et al.*, 1999). Therefore, more definitive answers on the frequency of mitotic checkpoint gene mutations in human tumors with chromosomal instability will have to await the results from additional screening studies.

B. Synergy between Mitotic Checkpoint Genes in Cancer Evolution

Regardless of the actual mechanism that causes the initial chromosomal mis-segregation event, it triggers a process that generates ever new and ultimately tumorigenic karyotypes (Duesberg and Rasnick, 2000; Duesberg *et al.*, 1999). Experiments with mice in which Rael and Bub3 are deleted individually or in combination suggest that mitotic checkpoint genes may act to regulate chromosomal instability rates in the evolution of cancer (Babu *et al.*, 2003). Unlike mice that are homozygous null for Rael or Bub3, mice that are double heterozygous for Bub3 and Rael are born alive. Although double heterozygotes have no overt abnormalities, cells from these mice exhibit much greater rates of premature sister chromatid separation and chromosome mis-segregation than single haplo-insufficient cells. These findings suggest that Bub3 and Rael act synergistically to prevent aneuploidy. It is therefore conceivable that the initial loss of a single mitotic checkpoint gene, for instance Rael, might start a vicious cycle in which reduced expression of that checkpoint protein causes additional chromosome loss. If that loss happened to involve mouse chromosome 7 containing the Bub3 gene locus, the rate of chromosomal instability would significantly increase. Because compound Rael/Bub3 heterozygotes seem to be more susceptible to DMBA induced lung tumor formation than the single heterozygous mice, it seems that the increased chromosomal instability accelerates tumorigenesis. However, once a tumorigenic karyotype has been established, preservation of this karyotype might provide a selective advantage. Perhaps by regaining a chromosome containing a mitotic checkpoint gene that is haplo-insufficient (for instance chromosome 7) cells might be able to reduce the chromosomal instability rate and preserve their tumorigenic karyotype. The model is summarized and presented in Fig. 2. Haplo-insufficient mouse models will prove useful in

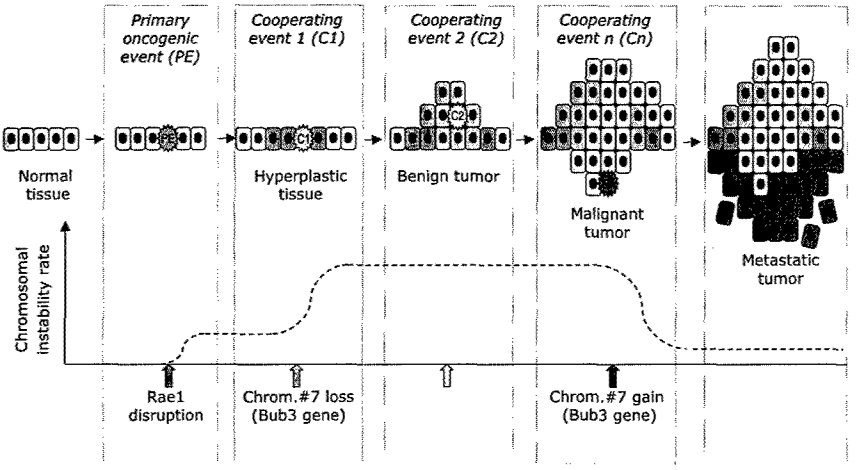

Figure 2 A model for how mitotic checkpoint genes may contribute to cancer evolution. Loss of a single mitotic checkpoint gene copy may drive a normal cell into a pathway of cancer (PE). Potential causes of such a loss include carcinogens that affect chromosome segregation, mitotic checkpoint gene mutations or epigenetic events that reduce mitotic checkpoint gene expression. The resulting checkpoint-defective cell may generate new karyotypes at relatively low rates. Further loss of a chromosome that contains another mitotic checkpoint gene (C1) may accelerate the mis-segregation rate and promote the formation of more tumorigenic karyotypes. Once a highly malignant karyotype has been established, preservation of this karyotype might be advantageous. One strategy for improving karyoptypic stability might be the regaining of lost chromosomes that contain mitotic checkpoint genes (see color plate).

elucidating whether the rate of chromosomal instability indeed declines at the end stages of cancer evolution.

C. Centromere-associated Protein Knockout Mice

Mad2, Bub3, and Rael mouse studies have taught us that the majority of cell divisions of checkpoint defective cells produce daughter cells with modal chromosome numbers. Thus, the mitotic checkpoint seems to function as a backup mechanism that prevents the occasional problem of mis-segregation from occurring in mitosis. However, the mitotic checkpoint machinery is likely to take on a more central role when key components of the chromosomal segregation machinery other than those involved in the mitotic checkpoint are altered by genetic or epigenetic events. Thus far, CENP-A, CENP-B, CENP-C, and CENP-E, have been studied in the mouse by gene disruption methods. CENP-B knockout mice are viable, have a normal lifespan and display no overt phenotype (Hudson *et al.*, 1998; Kapoor *et al.*, 1998; Perez-Castro *et al.*, 1998). In contrast, CENP-A

(Howman *et al.*, 2000), CENP-C (Kalitsis *et al.*, 1998) and CENP-E (Putkey *et al.*, 2002) knockout mice die during early embryogenic development. Like Mad2, Bub3, and Rael null embryos, CENP-A and CENP-E null embryos develop normally until the blastocyst stage but then fail to expand their inner cell mass and die. CENP-C null embryos start to degenerate a day earlier at the morula stage (16-cell stage). Probably CENP-A, CENP-C, and CENP-E knockout embryos develop normally in the earliest stages of development due to the presence of maternal gene products. However, once these products are depleted due to RNA and protein degradation processes, the consequences of the respective gene disruptions become apparent. The difference in onset of embryo degradation between CENP-C on the one hand and CENP-A and CENP-E on the other may merely result from differences in maternal product stability or protein level requirement, or both. Early embryonic death has hampered the phenotypic analysis of the CENP-A, CENP-C, and CENP-E knockout mice, but in all cases severe mitotic problems preceded the time of embryonic death. CENP-A null mice typically displayed micro- and macronuclei formation, nuclear bridging and blebbing, and chromatin fragmentation. CENP-C null mice exhibited similar features of chromosomal mis-segregation as CENP-A null mice. CENP-E loss produced metaphases with misaligned and/or centrophilic chromosomes due to unstable attachments between kinetochores and microtubules. CENP-E binds to the mitotic checkpoint protein BubR1 and its loss might inactivate the mitotic checkpoint. The high incidence of metaphases with lagging and centrophilic chromosomes suggests that CENP-E null cells are incapable of delaying mitosis despite the presence of unattached chromosomes, implying that the mitotic checkpoint is defective. It will be of interest to further test whether CENP-E deficient cells indeed will exit mitosis prematurely in the presence of spindle poisons such as nocodazole, just like cells that are insufficient for Mad2, Bub3, or Rael. It will also be of interest to analyze whether mice that are haplo-insufficient for CENP-A, CENP-C, or CENP-E exhibit increased chromosomal instability and carcinogen-induced tumor formation.

V. Conclusions and Future Directions

The goal of this review is to provide current mechanistic views of a highly complex process that ensures that cells with even a single unaligned chromosome will not prematurely exit mitosis (Fig. 3). These models are only possible because of the discovery of the molecular components of the spindle checkpoint, the kinetochore and the Anaphase Promoting Complex. Despite these advances, outstanding questions regarding all aspects of this signaling pathway remain unanswered. It remains to be determined how

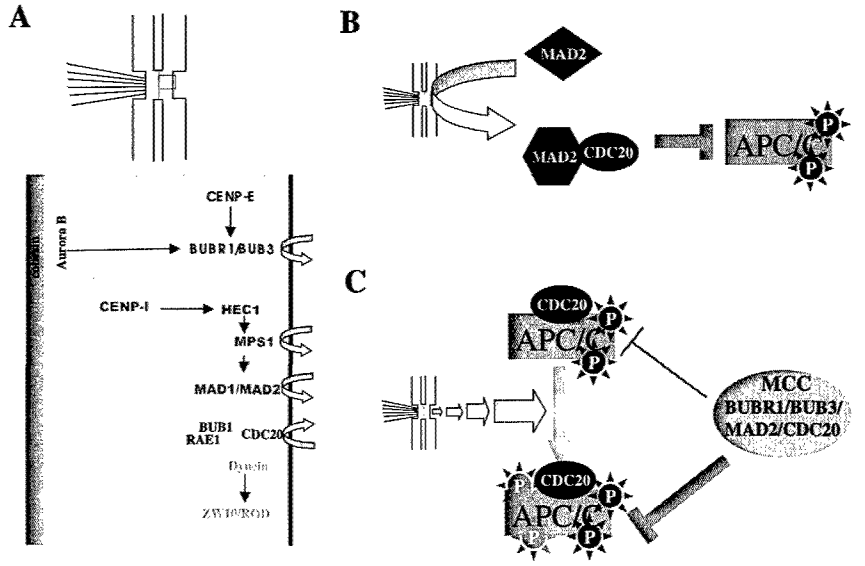

Figure 3 The mitotic checkpoint pathways in human cells. **A**. At least three redundant signaling pathways exist inside the unattached/tensionless kinetochore. The sensors (e.g., CENP-E) detect the absence of microtubule binding and/or tension, affect the behavior of some checkpoint proteins at the kinetochores and emit the "wait anaphase" signals. Several components recycling fast at kinetochores (curved arrows) may help the amplification of signals in the cytoplasm. The exact roles of several checkpoint proteins like BUB1, RAE1 in the kinetochores are still unknown. **B** and **C** are two models to explain how the signals originating from kinetochores lead to the inhibition of APC/C. **B**. Sequestration model. In this model, the unattached kinetochore facilitates a conformational change of MAD2 and results in its binding to CDC20. Sequestration of CDC20 from APC/C this way inhibits its ubiqutin ligase activity and prevents the anaphase onset. Several variants of this model exist now but what is common is they all prefer *de novo* formation of inhibitor(s) by unattached kinetochores to inhibit APC/C. **C**. APC/C sensitization model. In this model the inhibitory complex MCC exists throughout the cell cycle. The signals from unattached kinetochores may be amplified and lead to phosphorylation (or other modifications) of all the APC/C in the cytoplasm. This modified form of APC/C is sensitized and inhibited by MCC (see color plate).

checkpoint proteins monitor microtubule occupancy and tension at kinetochores (Fig. 3A). Although hBUBR1 is postulated to monitor CENP-E activities at kinetochores, this remains to be rigorously demonstrated. In addition, how are the microtubule binding activities of other proteins monitored? It is formally possible that each protein is assigned a different checkpoint protein. Alternatively, the different activities are relayed to a centralized detector that may be composed of multiple checkpoint proteins.

In addition to monitoring microtubule attachments, the sensor is also intimately linked to the generation of the "wait anaphase" signal.

The prevalent model which is based on rapid rate at which Mad2 binds and dissociates from unattached kinetochores suggests that Mad2 is the "wait anaphase" signal (Fig. 3B). Unattached kinetochores are believed to catalytically convert Mad2 into an altered state such that upon its release, the "activated" Mad2 can bind to CdC20 and sequester it from activating the APC/C. The discovery of the MCC has led to an alternative model whereby "wait anaphase" is not directly responsible for inhibiting the APC/C, but rather sensitizes the APC/C to prolonged inhibition by a cytosolic pool of inhibitor (Fig. 3C). Implicit in this model is that the "wait anaphase" signal must be amplified so that a single unattached kinetochore can target the large number of APC/C in the cell. The molecules involved in the amplification step is unknown but a reasonable prediction is a kinase cascade, perhaps mediated by some of the checkpoint kinases themselves. Finally, the mechanism by which MCC inhibits the APC/C is unknown, but a key is likely to lie in understanding how it distinguishes between mitotic vs interphase forms of the APC/C.

Separate from these mechanistic issues, the role of the spindle checkpoint in development and cancer remains a priority because of its direct connection to aneuploidy. In all cases examined so far, spindle checkpoint genes are essential for viability. This is clearly distinct from some DNA damage checkpoint genes like ATM where homozygous null mutants are viable. This difference may reflect the fact that chromosome segregation is inherently an error prone process whereby the checkpoint is essential for ensuring that mistakes are corrected. Thus, the accumulation of aneuploid cells during the earliest stages of embryogenesis may result in massive cell death or severe cellular defects that cannot sustain continued development. In the future, it will be interesting to generate conditional knockout mice so that it will be possible to test how a mature animal responds to the loss of the spindle checkpoint. One expectation is that these animals will develop tumors at frequencies that are higher than that seen for the haplo-insufficient mutants. This prediction is based on fundamental differences in the biochemical status of the spindle checkpoint between a null and a heterozygote mutant. In heterozygotes, a reduced level of a checkpoint protein might lower the overall output from an unattached kinetochore so that an unaligned chromosome cannot sustain a prolonged delay and cells exit mitosis prematurely. This contrasts with a null mutant where it may not be able to delay mitosis in response to the presence of unaligned chromosomes. Consequently, the null mutants will exit mitosis with unaligned chromosomes more frequently than a heterozygote. It is not difficult to imagine with 2.5×10^8 cells dividing in the human body at any given time that a small increase in the frequency of aneuploidy will accelerate tumor formation during the lifespan of an individual.

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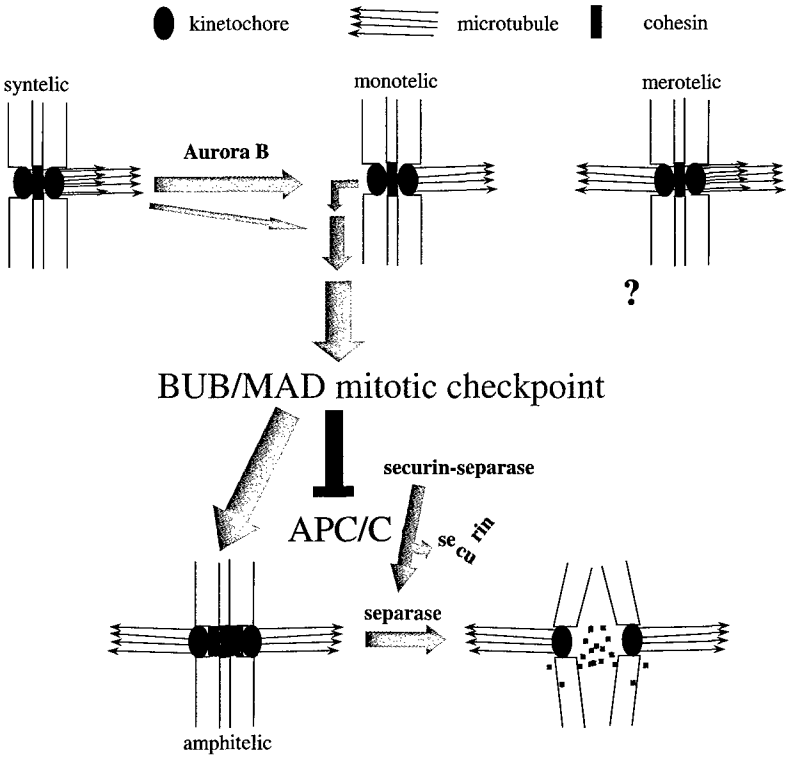

Figure 2.1 Mitotic checkpoint as a signal transduction pathway. Unattached kinetochores in monotelic chromosomes initiate "wait anaphase" signal transduction and activate mitotic checkpoint, leading to the inhibition of APC/C by BUB/MAD checkpoint proteins. The status monitored at kinetochores may be microtubule occupancy or tension. Syntelic chromosomes may be transformed into mototelic by Aurora B, or the lack of tension on their kinetochores may directly start the checkpoint. It is suggested merotelic attachment may not be able to activate the mitotic checkpoint. Only when all the chromosomes in a mitotic cell reach amphitelic attachment and tension develops between sister kinetochores will the mitotic checkpoint stop and APC/C catalyze the ubiquitination and degradation of securin. The released separase then cut the cohesion between sister chromatids, thus finishing the metaphase-anaphase transition. (Liu *et al.*, p. 32).

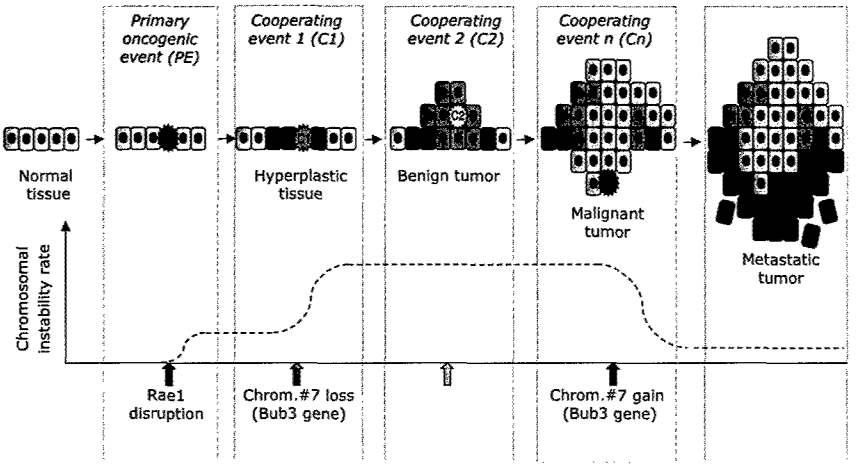

Figure 2.2 A model for how mitotic checkpoint genes may contribute to cancer evolution. Loss of a single mitotic checkpoint gene copy may drive a normal cell into a pathway of cancer (PE). Potential causes of such a loss include carcinogens that affect chromosome segregation, mitotic checkpoint gene mutations or epigenetic events that reduce mitotic checkpoint gene expression. The resulting checkpoint-defective cell may generate new karyotypes at relatively low rates. Further loss of a chromosome that contains another mitotic checkpoint gene (C1) may accelerate the mis-segregation rate and promote the formation of more tumorigenic karyotypes. Once a highly malignant karyotype has been established, preservation of this karyotype might be advantageous. One strategy for improving karyoptypic stability might be the of regaining lost chromosomes that contain mitotic checkpoint genes. (Liu *et al.*, p. 44).

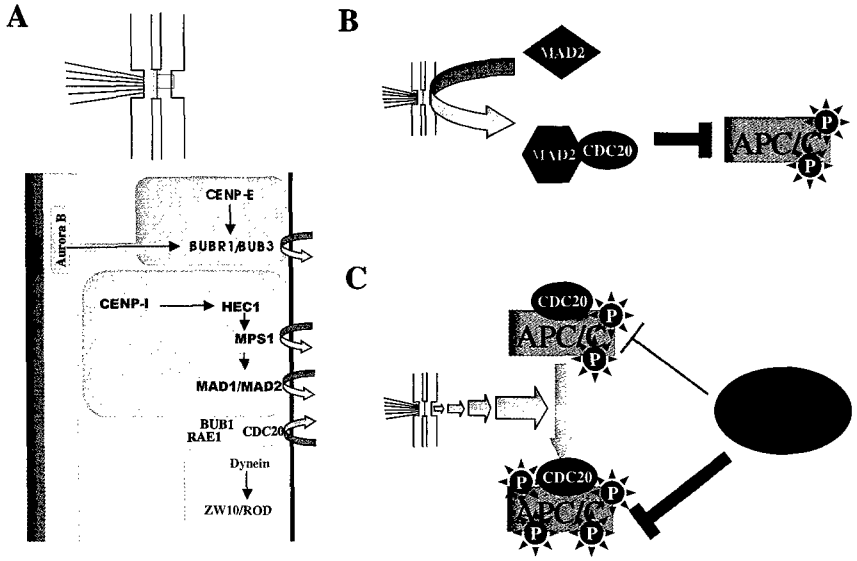

Figure 2.3 The mitotic checkpoint pathways in human cells. **A**. At least three redundant signalling pathways exist inside the unattached/tensionless kinetochore. The sensors (e.g. CENP-E) detect the absence of microtubule binding and/or tension, affect the behavior of some checkpoint proteins at the kinetochores and emit the "wait anaphase" signals. Several components recycling fast at kinetochores (curved arrows) may help the amplification of signals in the cytoplasm. The exact roles of several checkpoint proteins like BUB1, RAE1 in the kinetochores are still unknown. **B** and **C** are two models to explain how the signals originating from kinetochores lead to the inhibition of APC/C. **B**. Sequestration model. In this model, the unattached kinetochore facilitates a conformational change of MAD2 and results in its binding to CDC20. Sequestration of CDC20 from APC/C this way inhibits its ubiqutin ligase activity and prevents the anaphase onset. Several variants of this model exist now but what is common is they all prefer *de novo* formation of inhibitor(s) by unattached kinetochores to inhibit APC/C. **C**. APC/C sensitization model. In this model the inhibitory complex MCC exists throughout the cell cycle. The signals from unattached kinetochores may be amplified and lead to phosphorylation (or other modifications) of all the APC/C in the cytoplasm. This modified form of APC/C is sensitized and inhibited by MCC. (Liu *et al.*, p. 46).

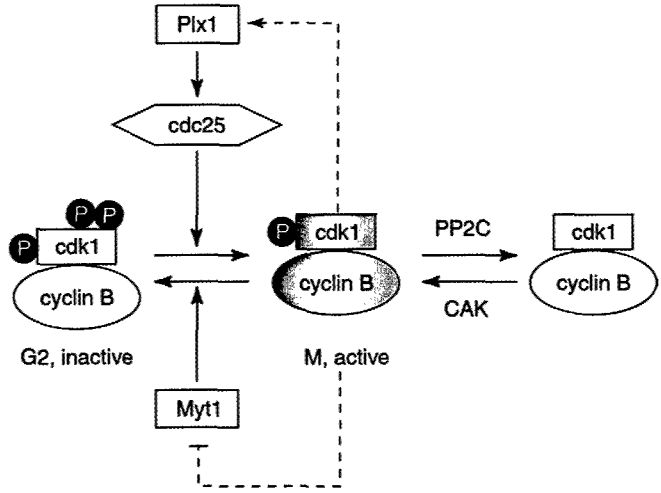

Figure 3.3 Regulation of cdk1 activation. Activation of cdk1 depends on association with cyclin B and the phosphorylation of cdk1 protein within the cdk1-cyclin B complex by cdk activating kinase, CAK. Protein phosphatase 2C (PP2C) can reverse the CAK-mediated phosphorylation. Active cdk1-cyclin B can be inactivated by the Myt1 protein kinase present in the oocyte, whose effects are reversed by the activating phosphatase cdc25. Plx1—polo-like kinase 1, which phosphorylates and activates cdc25. Positive feedback loops are shown as dashed lines. The figure is adapted from Ferrell, 2002. (Voronina and Wessel, p. 71).

Crystal Structure of the Motor Domain of the Human Kinetochore Protein CENP-E

Isabel Garcia-Saez, [$]Tim Yen, Richard H. Wade, Frank Kozielski

Laboratoire de Microscopie Electronique Structurale, Institut de Biologie Structurale (CEA-CNRS-UJF), 41, rue Jules Horowitz, 38027 Grenoble Cedex 01, France,

[$]Institute for Cancer Research, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, PA 19111, USA.

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Abstract

The human kinetochore is a highly complex macromolecular structure that connects chromosomes to spindle microtubules in order to facilitate accurate chromosome segregation. CENP-E (Centromere-Associated Protein E), a member of the kinesin superfamily, is an essential component of the kinetochore since it is required to stabilize the attachment of chromosomes to spindle MTs, to develop tension across aligned chromosomes, to stabilize spindle poles and to satisfy the mitotic checkpoint. Here we report the 2.5 Å resolution crystal structure of the motor domain and linker region of human CENP-E with MgADP bound in the active site. This structure displays subtle but important differences compared to the structures of human Eg5 and conventional kinesin. Our structure reveals that the CENP-E linker region is in a "docked" position identical to that in the human plus-end directed conventional kinesin. CENP-E has many advantages as a potential anti-mitotic drug target and this crystal structure of human CENP-E will provide a starting point for high throughput virtual screening of potential inhibitors.

Introduction

During cell division, chromosomes capture spindle microtubules and congress to the spindle equator. They then separate by moving towards the spindle poles to provide each daughter cell with the same set of genetic information. A highly regulated macromolecular complex, called the kinetochore, connects chromosomes to spindle microtubules. Significant progress has been made in recent years to identify kinetochore-associated proteins and to elucidate their roles during mitosis, reviewed in (Yen and Schaar 1996; Rieder and Salmon 1998; Grancell and Sorger 1998; Kapoor and Compton 2002; Cleveland *et al.* 2003). Among many other proteins, kinetochore-associated motor proteins such as dynein (Steuer *et al.* 1990; Pfarr *et al.* 1990) and members of the kinesin family, CENP-E (Centromere-Associated Protein E) (Yen *et al.* 1991; Yen *et al.* 1992) and MCAK (Mitotic Centromere-Associated Kinesin) (Wordemann and Mitchison 1995) have been shown to be involved in essential mitotic events.

CENP-E was first discovered in human cells by a monoclonal antibody that was raised against chromosome proteins that were enriched for known centromere/kinetochore components (Yen *et al.* 1991) Subsequently, CENP-E was found to be a novel member of the kinesin superfamily (Yen *et al.* 1992). Since the initial discovery, CENP-E has been identified in *Xenopus laevis* (Wood *et al.* 1997), *Drosophila melanogaster* (Yucel *et al.* 2000) and genome sequence analysis has identified putative homologues in *Mus musculus* (Miki *et al.* 2001) and in *Arabidopsis thaliana* (Dagenbach and Endow 2004).

CENP-E expression in human cells is cell-cycle-dependent. It is low in early G1 but increases as cells progress through the cell cycle and peak levels are detected during late G2 and mitosis (Yen *et al* 1991; Yen *et al* 1992; Brown *et al.* 1994). Despite its presence throughout the cell cycle, CENP-E is not detected at kinetochores until early prometaphase and remains there until anaphase A, albeit at significantly reduced levels. By anaphase B, CENP-E is also localized to the interzonal microtubules of the mitotic spindle. In telophase cells, CENP-E is concentrated at the midbody until it is eventually degraded quantitatively at the end of mitosis through a cytokinesis-independent mechanism (Brown *et al* 1996).

Disruption of CENP-E functions by antibody microinjection, transfection of dominant-negative mutants, anti-sense or RNA_i and gene knockouts has shown that it is essential for

some aspects of kinetochore microtubule attachments. (Yen et al. 1991; Schaar *et al.*1997; Yao *et al.* 2000; Chan *et al.*; 2002; Weaver *et al.* 2003]. CENP-E appears to be essential for monopolar chromosomes to establish bipolar attachments. As the unattached kinetochore of a monopolar chromosome does not encounter microtubules that emanate from the opposite pole at high frequencies, CENP-E is thought to enhance the efficiency by which kinetochores establish stable microtubule attachments. In contrast to monopolar chromosomes, chromosomes that are situated in the center of the spindle are able to establish bipolar attachments as the higher frequencies of microtubule encounters are thought to compensate for the loss of CENP-E. Quantitative EM studies revealed that bipolar kinetochores lacking CENP-E are capable of establishing near normal numbers of microtubule attachments (McEwen *et al.* 2001). These bipolar connections are nevertheless defective as they are unable to generate sufficient poleward force to achieve normal levels of tension between the sister kinetochores (Yao *et al.* 2000; McEwen *et al.* 2001).

Kinesins belonging to the CENP-E subfamily are significantly bigger than all other members of the superfamily. Human CENP-E is composed of 2663 residues and has three distinct domains : an N-terminal motor domain (residues Met1-Lys327) that includes MT and ATP binding sites, a long discontinuous α-helix (residues Asn336-Ala2471) and a C-terminal MT-binding (Liao *et al.* 1994) domain (residues Gln2472-Gln2663). The kinetochore-binding region is located in the C-terminal part of the protein (residues Ile2126-Val2476) (Chan *et al.* 1998). Human CENP-E contains two regions with homology to PEST sequences (residues Arg459-Lys489 and His2480-Lys2488), which might be responsible for rapid intracellular degradation of CENP-E at the end of mitosis (Brown *et al.* 1994). The carboxy-terminal ATP-independent MT binding site (residues Glu2565-Gln2663) in human CENP-E is thought to be regulated *in vivo* by mitotic phosphorylations that inhibit microtubule binding. The presence of several consensus phosphorylation sites for a cyclin B cdc2 kinase complex is consistent with the ability of this kinase to phosphorylate and inhibit microtubule binding by this domain *in vitro* (Liao *et al.*1994). In addition, the *in vivo* association of CENP-E with MAP (Mitogen Acitivated Protein) kinase during mitosis suggests there may be an additional regulation mechanism for the interaction between microtubules and chromosomes and thus mitotic progression (Zecevic *et al.* 1998).

Previous work has established that the critical determinant that specifies the directionality of kinesins along microtubules is a short region, that we refer to as the linker

(sometimes called neck), linking the kinesin motor domain to the α-helical coiled region, for short reviews see (Endow and Fletterick 1998; Woelke and Schliwa 2000). This linker region is found to be distinctly different between plus-ended kinesins whose motor domains are located near the N-terminal end of the polypeptide chain and minus-ended kinesins whose motor domains are located near the C-terminus. In the case of CENP-E the current situation is somewhat confusing since there have been reports of slow plus-end directed movement (Wood *et al.* 1997), of slow minus-end directed motor activity (Thrower *et al.* 1996) or simply of microtubule tethering without movement (DeLuca *et al.* 2001]. These discrepancies, along with the considerable importance of human CENP-E, have encouraged us to engage structural studies, and we describe here the first crystal structure of the motor domain of this kinetochore-associated protein.

Materials and Methods

Construction of plasmids for protein expression. The DNA construct coding for the human CENP-E motor domain was synthesized by PCR, using the following forward and reverse primers CENP-E_1 : 5'-CCA GTT CAG CCT GAT ACC ATG GCG GAG GAA GGA GCC, and CENP-E_2 : 5'-ATA CCT TTT CAG GAG CTC GAG ATC AGT TGA TAC CTC. The PCR product as well as expression vector pET28a were double-digested with NcoI and XhoI and ligated. Positive expression clones were identified by testing for the presence of an insert of the expected size by digesting the purified plasmids with the restriction enzymes mentioned above. The sequence was confirmed by DNA sequencing. The expression clone codes for the CENP-E motor domain and linker region (residues Met1-Glu342) and eight additional residues (LEHHHHHH) at the C-terminus of the protein.

Expression and purification of CENP-E. Recombinant CENP-E was expressed and purified as described for monomeric human Eg5 (DeBonis *et al.* 2003). CENP-E is unstable and consequently the protein was freshly prepared for crystallization assays.

Protein crystallization. For crystallization, CENP-E in 20 mM Pipes 7.3, 200 mM NaCl and 1 mM EGTA was supplemented with 2 mM ATP and 10 mM $MgCl_2$ and concentrated (AMICON ULTRA-15, 30 kD) to 11 mg/ml. Insoluble material was removed by centrifugation at 30 000 g for 15 min. Sitting drops (1 µl protein : 1 µl reservoir) at 19°C were set up with freshly purified protein using a TECAN crystallization robot and 15 commercial

kits (Hampton Research). The detailed automated crystallization procedure is described elsewhere (Garcia-Saez, submitted). Crystals appeared after two days in different crystallization conditions. Long rods were obtained after manually improving the initial conditions using 1 µl of CENP-E at 11 mg/ml and 1 µl of reservoir solution containing 23% PEG 3350, 0.2 M $NaNO_3$, 0.1 M Pipes 7.0, in hanging drops at 19°C. Crystals belonged to space group $P2_1$ with unit cell parameters a = 49.35 Å, b = 83.70 Å, c = 94.16 Å and monoclinic angle β= 103.05°. The solvent content was calculated to be 42% assuming 2 molecules per asymmetric unit.

Data collection. 4 different native data sets were collected at beamline ID14-2 of the European Synchrotron Radiation Facility (ESRF), equipped with an ADSC Quantum-4 CCD detector and processed with the DENZO/SCALEPACK program suite (Otwinowsky and Minor 1997) as well as SCALA from the CCP4 package (Collaborative Computational Project 1994). The observed diffraction patterns were highly anisotropic with a resolution better than 2.0 Å in one direction but worse in the other. The best dataset yielded data to 2.5 Å resolution with a completeness of 98%. More details of data collection and processing are given in Table 1.

Structure determination and refinement. The CENP-E motor domain structure was solved by molecular replacement using AMoRe (Navaza and Saludjian 1997). The structure of conventional human kinesin, Protein Data Bank code 1MKJ (Sindelar *et al.* 2002) without ions, ADP or water molecules was used as a starting model. The correct solution, after performing a two molecule/asymmetric unit search, yielded a correlation coefficient of 39.8% and an R factor of 47.8 %. After an initial round of rigid-body refinement, the model was rebuilt manually using TURBO-FRODO (Roussel and Cambillau 1991). MgATP was included at the initial stages. The model was further refined by cycles of simulated annealing, energy minimization and B-factor refinement using CNS (Brünger *et al.* 1998) and subsequent manual model building. In early stages of refinement, non-crystallographic symmetry restraints with decreasing restraint weights were used, but in the later stages both monomers were considered to be independent. Water molecules were added progressively during refinement. The quality of the model was accessed with PROCHECK (Laskowski *et al.* 1993). Residues for which no electron density was visible were omitted from the model. The occupancy of the side chains of the following residues in monomer A were set to 0.00: Asn136, Glu137, Arg202, Arg251. Residues Lys32, Phe125 and Ser261 were in double

conformation. The occupancy of the side chains of the following residues in monomer B are set to 0.00: Gln115, Arg202, and Gln267. 78.6% of all residues are in most favoured, and 16.2% in additionally allowed regions. 1.4% are in disallowed regions. Final refinement statistics are given in Table 1.

Preparation of figures. Figure 1 was generated using TURBO-FRODO (Roussel and Cambillau 1991). Figures 2, 6 and 7 were prepared using Molscript (Kraulis 1991), figure 4 was done with BOBSCRIPT (Esnouf, 1999). Structural sequence alignment in figure 3 was performed using the program ESPRIPT (Gouet *et al.* 1999) and adjusted by hand. Figure 5 was prepared using GRASP (Nichols 1992).

Results

A construct with the amino-terminal 342 residues of CENP-E that contains the ATP sensitive microtubule binding site along with the ~12 residue linker region was expressed in *E. coli*. N-terminal sequencing of the first 7 residues revealed that the first methionine is missing (peptide sequence: AEEGAV) due to bacterial processing (Hirel et al. 1989). The measured molecular mass of 39149 D using electrospray mass spectrometry is in excellent agreement with the predicted mass of 39179 D. Gel filtration data (not shown) suggests that CENP-E is monomeric.

The crystal form investigated has two CENP-E molecules (A and B) per asymmetric unit. We ask whether these are indeed two independent monomers ? The relative orientations of the two motor domains in the crystal structures of established kinesin dimers, i.e. *R. norvegicus* conventional kinesin (Kozielski *et al.* 1997) and *D. melanogaster* ncd (Kozielski *et al.* 1999], are notably different to those in the asymmetric unit of our CENP-E crystals, figure 1. Since our construct does not extend into the accepted dimerization domain, this suggests that in the present case there are two independent monomers whose relative orientations are due to crystal packing and electrostatic interactions. The residues involved are listed in Table 2. In monomer A, they localise to the end region of β1c, to α4 and to the C-terminal loop between α6 and the linker region. In monomer B, they are near the N-terminus, mainly in β1a, β1b and β1c (table 2).

The resolved structure of monomer A includes residues Glu4-Lys216, Gly224-Ala243, Arg251-Ser339, bound MgADP and a molecule of Pipes (1,4-piperazinediethanesulfonic acid) that was present in the crystallization buffer. The final model in monomer B comprises residues Gly5-Asn17, Glu21-Lys216, Thr225-Ala243 and Leu252-Ser339 and bound MgADP. When 304 C_α positions in the two monomers were superposed by a least-squares fit, their final r.m.s. deviation after three cycles was 0.72 Å. Only monomer A results are presented here since it's electron density map is clearly better than that of monomer B (Figure 2). The final refined model contains 77 water molecules.

Figure 2, a and b, shows front and back views of the CENP-E motor domain structure. It has has a mixed eight stranded β-sheet core with flanking solvent exposed α-helices and a small three stranded antiparallel β-sheet in the N-terminal region. Interestingly, the linker region, figure 2c. (residues Tyr328-Ser339), has the same docked conformation as found in *R. norvegicus* KHC (Kozielski *et al.* 1997; Sack *et al.* 1997) and in one of the *H. sapiens* KHC structures (Sindelar *et al.* 2002). Residues in β9 and β10 in the linker form main chain hydrogen bonds with β8 and β10 respectively in the motor domain core giving short anti-parallel β-sheets between β9-β8 and β7-β10. The following amino acid residues are involved : Asn336 N and the O of Gly77, O of Asn337 with Val228 N, Val338 N with V226 O, and V338 O with V226 N. There are also electrostatic interactions between the side chains of Asn336 and Asn79. A water molecule, Wat60, plays a structural role in the stabilization of the "cross-road" between the residues of the motor domain and the linker by forming hydrogen bonds with Asn79 N (located in the N-terminal part of β3), Asn299 ND2 (located in loop 13 after α5) and the linker Tyr334 O and Asn336 OD1.

MgADP and three water molecules are located in the nucleotide binding pocket (Figures 2 & 4a). The Mg ion interacts with two β-phosphate oxygen moieties, with three water molecules and with the hydroxyl moiety of Thr93 at the end of the P-loop motif. The interactions between MgADP and specific amino-acid residues in the pocket are listed in table 3. The expected position for γ-phosphate is empty. Curiously, a molecule of Pipes, from the buffer, is located in pocket some 15.3 Å away (Figure 5). The bottom of this pocket is formed by the beginning of helix α5, and the "walls" by a turn between β4 and β5 and the N-terminal part of helix α4. A residue that has a double conformation, Ser261, contacts the Pipes molecule.

Discussion

CENP-E is a very important component of the kinetochore during mitosis where it is essential both for the stable bi-oriented attachment of chromosomes to spindle microtubules and for chromosome movements leading to congression. Currently, however, the reported interactions with microtubules are largely ambiguous and raise the following question. Can this kinesin support directed movement along microtubules and is this compatible with reported chromosome transport towards the minus ends of depolymerising microtubules (Lombillo *et al.* 1995).

To determine whether there is some unique structural explanation that may resolve the variable accounts of CENP-E motor activity, we have solved the crystal structure of the CENP-E motor domain. The linker region, with the two short β strands, β9 and β10 (Tyr228 - Ser339) is particularly interesting. This region has the same docked conformation found for other N-terminal motor domain kinesins, human KHC and Eg5 (Turner *et al.* 2001) and rat KHC. From the structural point of view, therefore, CENP-E appears to have all the features of a plus-end directed kinesin: the motor domain is in the N-terminal region of the polypeptide chain and it has a linker region conforming to the N-type kinesin model. This is consistent with the plus-end activity exhibited by Xenopus CENP-E. Nevertheless, the directionality of human CENP-E remains to be verified by *in vitro* motility assays using expressed dimer constructs.

Another interesting feature is that linker docking has been reported as a consequence of the presence of sulfate ions, mimicking inorganic phosphate, in specific cavities close to the nucleotide binding site (Sindelar *et al.* 2002). In the present case no sulfate was present in the crystallisation buffer and no density is visible in the same cavities. Consequently, the hypothetical role of the 'phosphate cavities' for linker docking appears to be in question.

A large number of kinesins are now known and this superfamily has ten or more phylogenetic subgroups as established by detailed comparisons of their motor domain amino-acid sequences (Dagenbach and Endow 2004). Each subgroup appears to be associated with a specific function at different stages of the cell cycle. Apart from the criterion of overall sequence similarity, each subfamily is also clearly characterized by specifically located

sequence insertions and deletions (Wade 2002). Thus, it appears that subfamily specific functionality may be related to subtle structural differences introduced by these insertions and deletions. The two other motor domain structures of kinesin superfamily members in *H. sapiens* that have been determined are conventional kinesin (Sindelar *et al.* 2002), involved in intracellular transport, and Eg5 (Turner *et al.* 2001) responsible for stabilization of the bipolar spindle in mitosis (Blangy *et al.* 1995). Since human CENP-E, Eg5 and KHC belong to distinct phylogenetic subgroups, we have compared their structures so as to visualize subfamily specific structural features, (Figures 3 and 6).

The sequence identity between the motor domains of CENP-E and KHC is 38.0% and the r.m.s. deviation between their crystal structures is 1.1 Å after least squares alignment of 284 C_α atoms. The identity between CENP-E - Eg5 is 36.1% and the rms deviation is 1.5 Å for least squares alignment of 256 C_α atoms. Compared to KHC, CENP-E has the following subfamily specific insertions and deletions: a three residue insert in loop 2, a three residue deletion in loop 5, a five residue insert in loop 10, a two residue insert in loop 12, a two residue deletion at the beginning of α6 (Figure 3). Particularly striking is the loop 2 between β1b and β1c, slightly longer than in KHC. This loop is oriented perpendicular to the equivalent loop in Eg5 that has a long insertion. Helix α2 is interrupted by loop 5 in all kinesin motor domain structures so far resolved. This loop is only seven residues long in CENP-E, which is shorter than for any other N-terminal motor domain kinesin. Interestingly, in Eg5 this loop has a seven residue insert compared to KHC and this insert is characteristic of the subfamily (this subfamily is also known as BimC or N2). It is not known whether this region has any specific function as it is on the opposite face to the commonly accepted microtubule interaction region. Another disordered region in CENP-E is the "tip" of the arrow-shaped structure, the loop 10 between β6 and β7. This region, from lysine 216 to serine 225, is invisible in our electron density map. In CENP-E this loop has 5 additional residues compared to KHC and it is probably highly flexible since it is visible in both the KHC and Eg5 structures. Finally, at the C-terminal end of the motor domain core structure, the helix α6 is two residues shorter in human CENP-E, and in other members of the subfamily, than in any other kinesin.

The active site structures of human KHC, Eg5 and CENP-E are highly conserved. The three water molecules in the CENP-E nucleotide binding pocket that coordinate with ADP

and the Mg ion are also found in the Eg5 and KHC structures (Figure 4). Nevertheless, conserved Arg14 and Pro15 of the N-4 motif (residues Arg12-Pro15), described to be involved in the binding of the purine moiety, are in different positions in CENP-E, since loop 1 immediately after β1 (from Leu16) shows high flexibility and is positioned differently compared to KHC and Eg5 which both contain two turn α-helices in this region (Figure 7). Particularly, the Arg14 side chain interacts with the oxygen in the ribose ring (Figure 4a).

The phosphate-binding loop of CENP-E (P-loop, or motif N-1) (residues Gly86-Thr93), involved in the interaction with α– and β-phosphates of the nucleotide, is structurally conserved in the three structures (Figure 7). Concerning the nucleotide state sensing areas, switch 1 (N-2, residues Asn197-His204) the nucleotide binding motif of the ATP γ-phosphate, appears to be in slightly different position to Eg5 and KHC (Figure 6) mainly at Asn197 and Gln198 since in CENP-E the helix α3a region immediately before these residues is disordered. Nevertheless, switch 1 is similar in the three structures. Switch 2 (N-3 motif) is also involved in binding the γ-phosphate. It is located immediately after β7 (residues Asp235 - Glu240) and differences in this region in CENP-E occur at the beginning of loop 11.
Helix α4 in the switch 2 cluster is in an up conformation that is correlated with the docking of the CENP-E linker as in human and rat KHC (Sindelar *et al.* 2002; Kozielski *et al.* 1997).

As previously described for the mitotic kinesin human Eg5 (Blangy *et al.* 1995; Mayer *et al.* 1999; Bergens *et al.* 2002; Johnson *et al.* 2002), the discovery of specific inhibitors of the kinetochore-associated CENP-E has considerable interest for future anti-mitotic therapies. The advantages of human CENP-E as a potential drug target have been recently reviewed (Miyamoto *et al.* 2003; Jablonski *et al.* 2003). These include its apparently complete degradation at the end of the mitotic event (Yen *et al.*1992), and the absence of any additional role of CENP-E in interphase. The crystal structure of human CENP-E will therefore provide a starting point for high throughput virtual screening of potential inhibitors and as the basis for the structure determination of future CENP-E - inhibitor complexes.

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Accession Numbers

Crystallographic coordinates and structure factors for the *H. sapiens* CENP-E motor domain structure have been deposited with the Protein Data Bank under code xxx.

Figure Legends

Figure 1: Crystal packing of the two CENP-E monomers within the asymmetric unit. The Cα model of monomer A (grey) and monomer B (rose) are shown. Residues interacting in the interface between monomers A and B are colored in red and green, respectively.

Figure 2: Stereoplot of the CENP-E motor domain structure. β-strands are colored in green, α-helices in blue and loops in yellow. The linker region (containing β9 and β10) is colored in red. Bound MgADP in the nucleotide-binding pocket is displayed as a stick and ball model. The numbering of the secondary structure elements is that used by Kull and coworkers (Kull *et al.* 1996). A) Front view. B) Back view, rotated 180° with respect to A.

Figure 3: Structural and sequence alignment of the three known human kinesin structures, CENP-E, Eg5 and conventional kinesin. Identical residues are colored in white with a red background, similar residues in red. The position of the regions forming the nucleotide-binding pocket (N-1 to N-4) as well as the position of the neck region are indicated in the figure.

Figure 4: Structural comparison of the nucleotide binding site of human a) CENP-E b) KHC and c) Eg5. The electron density for MgADP is depicted for CENP-E. The residues of the P-loop are indicated in red. Three conserved water molecules in contact with MgADP are labeled.

Figure 5: GRASP representation of CENP-E surface with bound ADP and Pipes. The closest distance between the two molecules is 15.3 Å.

Figure 6: Comparison of the motor domain crystal structures of three human kinesins, Eg5, CENP-E and KHC.

Figure 7: Superposition of the P-loop area structures of human KHC (coil in yellow), Eg5 (coil in green) and CENP-E (coil in white). The MgADP is taken from the CENP-E structure. Discuss difference in N-4 motif.

Tables

Table 1 : X-ray Data Collection Statistics[a] and Structure Refinement of the CENP-E motor domain

Unit cell dimensions	a = 49.35Å
	b = 83.70Å
	c = 94.16Å
	β = 103.05°
Space group	$P2_1$
Molecules per asymmetric unit	2
Maximum resolution (Å)	2.5
N° of unique reflections	25698
Overall completeness (%)	98
Last shell completeness (%)	86[b]
Multiplicity	4.7
R_{sym}[c]	0.064 (0.158)[b]

Refinement Statistics

N° of reflections	24415
$R_{working}$[d] (%)	23.00
R_{free}[e] (%)	28.33
Rms deviation from ideal	
Bonds (Å)	0.0078
Angles (°)	1.73217

[a]Data collection obtained on the ID14-2 X-ray beamline at the European Synchrotron Radiation Facility (ESRF) in Grenoble (France). [b] Last resolution shell : 2.64-2.50Å. [c] $R_{sym} = \sum|I_j - \langle I \rangle| / \sum \langle I \rangle$, where I_j is the intensity for reflection j, and $\langle I \rangle$ is the mean intensity. [d] $R_{working} = \sum||F_o| - |Fc|| / \sum|Fc|$, calculated with the working set. [e] R_{free} was similarly calculated with 4.5% of the data excluded for data at 2.5 Å.

Table 2: Interactions between monomers A and B in the asymmetric unit of CENP-E crystal (distances ≤ 3.5 Å)

A / B	N48 ND2	K270 NZ	S273 OG	D274 OD1	D274 OD2	D274 O	K327 NZ	Y328 CG	Y328 CD2	Y328 CE2	Y328 OH	K330 NZ
D42 OD1	2.69											
D34 OD2		2.77										
Y39 OH			3.11									
N35 ND2				2.85								
Y39 OH					2.57							
N48 ND2						3.32						
G43 O							3.32					
Y39 CD1								3.36*				
Y39 CD1									3.37*			
K45 O										3.17		
Q40 O											2.85	
S44 O												3.39

*: indicates hydrophobic interactions between the side chains of Tyr328 and Tyr39.

Table 3: Main ADP interactions with nucleotide binding site of CENP-E (distances ≤ 3.5 Å)

	ADP atoms											
CENP-E	**PB**	**O1B**	**O2B**	**O3B**	**O1A**	**O2A**	**O3A**	**C4***	**O4***	**C6**	**N6**	**N1**
A89 N	3.36			2.83			3.40					
S90 N		3.44										
G91 N		3.23										
K92 N		2.85										
K92 NZ		3.07										
T93 N			2.94		3.38							
T93 OG1			2.83									
Y94 N					2.92							
R14 NH2								3.46	3.06			
P15 CD										3.46*		
Y94 CE1										3.48*		
R12 NH2											3.28	
Y94 CZ												3.49
Mg	3.44		2.50	3.17								
Wat14			2.99			2.58						
Wat53												3.24
Wat55				3.33								

*: indicates hydrophobic interactions

Figures

Figure 1

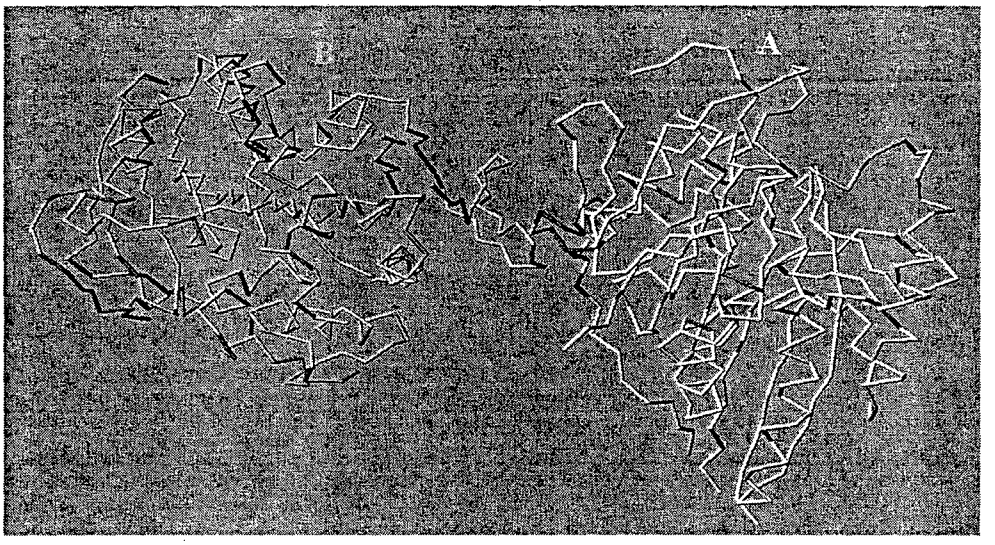
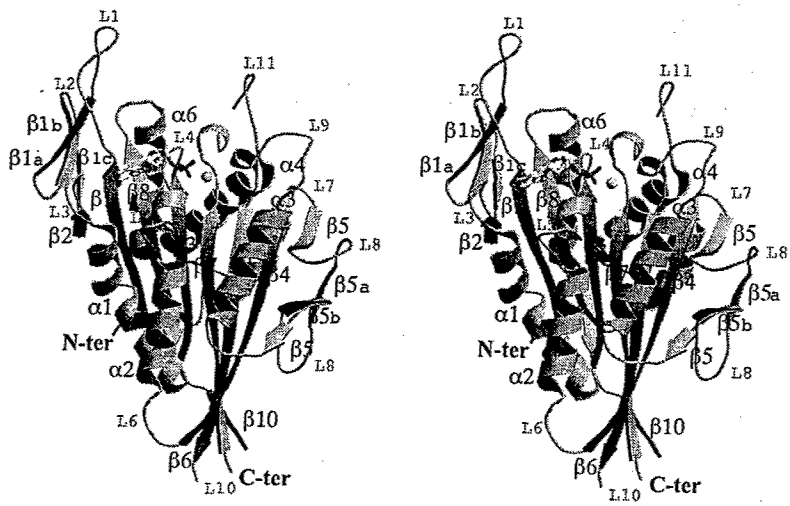


Figure 2

A)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L4
L9
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β1c
α4
L7
β
β8
α3
L3
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β2
L8
β4
β5a
α1
β5b
N-ter
β5
α2
L8
L6
β10
β6
C-ter
L10
L1
L11
L2
α6
β1b
L4
L9
β1a
β1c
α4
L7
β
β8
α3
L3
β5
β2
L8
β4
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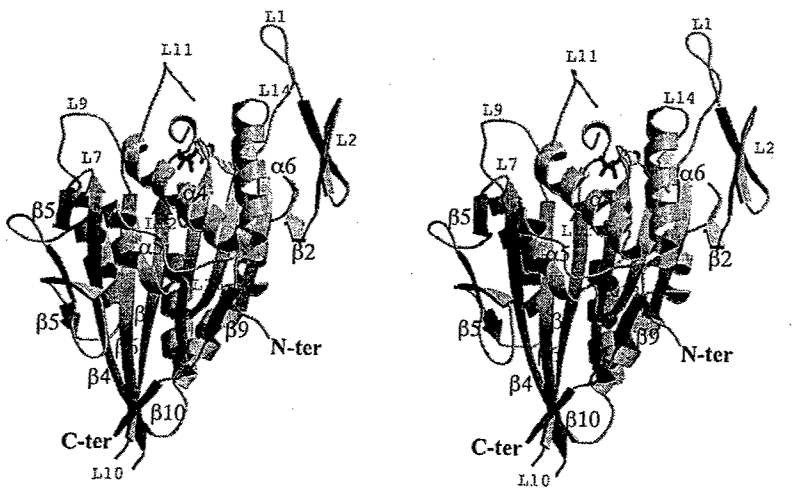
L6
β10
β6
C-ter
L10

B)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β5
β2
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β9
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β4
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L10
L1
L11
L14
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β4
β10
C-ter
L10

Figure 3

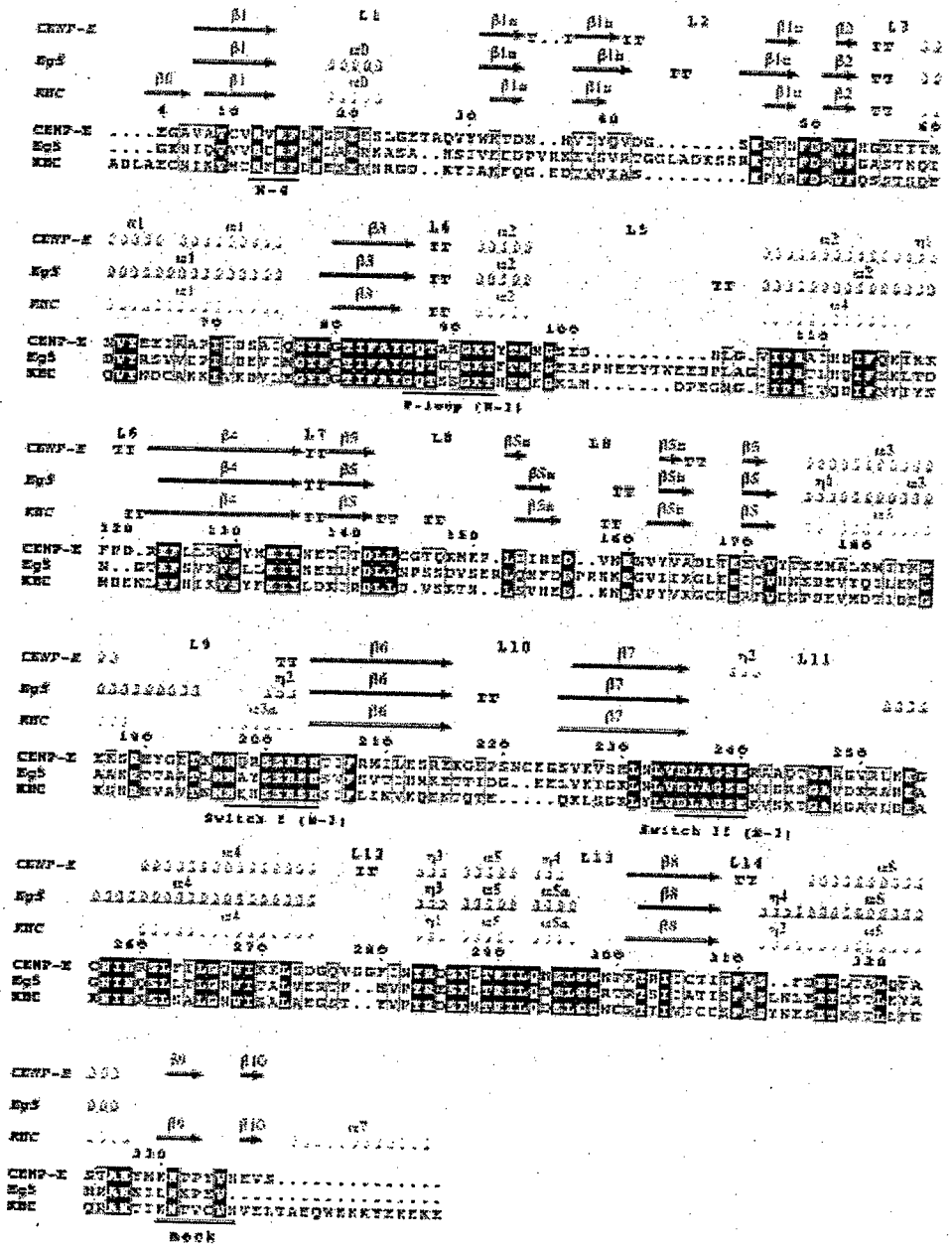
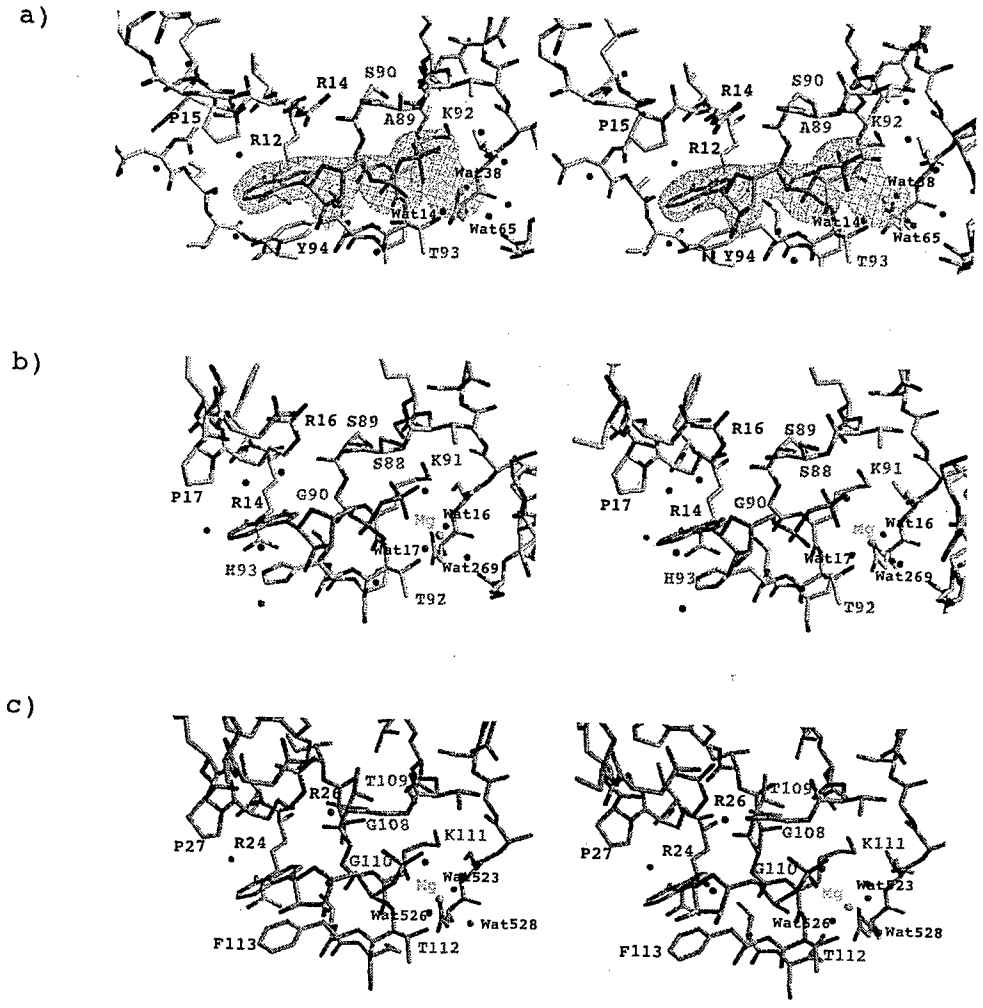


Figure 4


a)
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S90
P15
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K92
R12
Wat38
Wat14
Wat65
Y94
T93
b)
R16
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S88
K91
P17
R14
G90
Wat16
Wat17
Wat269
H93
T92
c)
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T109
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T112

Figure 5

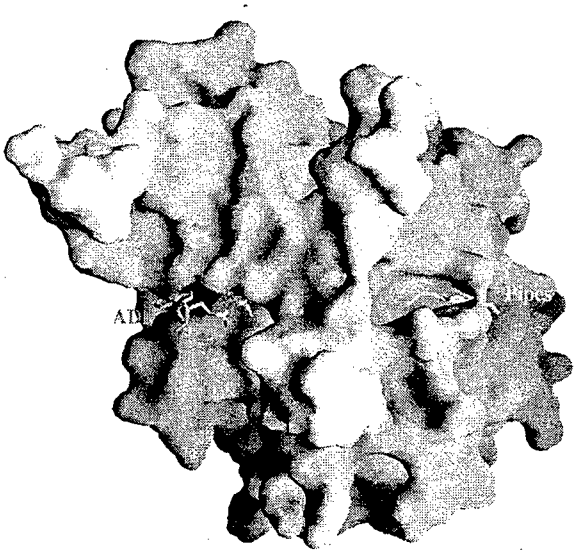
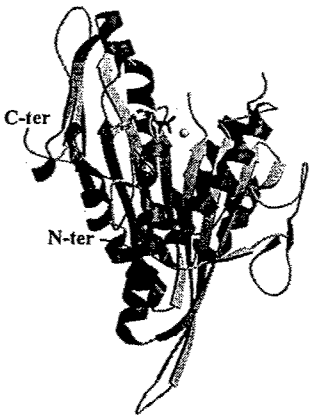
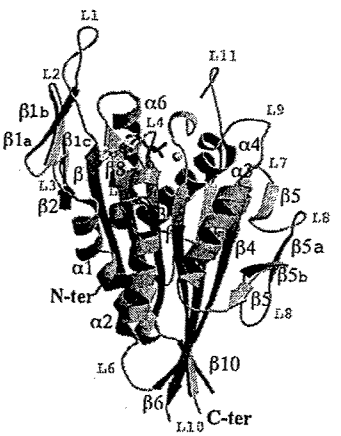


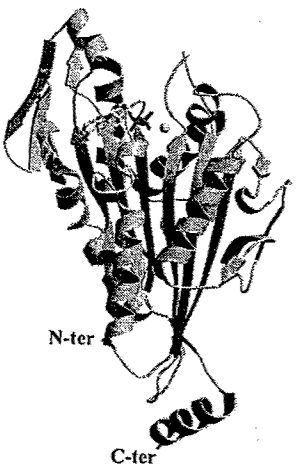
Figure 6



HsEg5



HsCENP-E



HsKHC

Figure 7